

MGIPC-S4-10 AR-21-6-48-1,000.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER
MEMORIAL FUND

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VOLUME 175
BALTIMORE
1948

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THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, INC.
WAVERLY PRESS, INC.
BALTIMORE 2, U. S. A.

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CORRECTION

On page 90, Equation 2, Vol. 136, No. 1, October, 1940, read

" $\Sigma d^2 = \Sigma [W - k_1 G - k_2 L - k_3 P]^2$ " for " $\Sigma d^2 = [\Sigma W - k_1 \Sigma G - k_2 \Sigma L - k_3 \Sigma P]^2$."

THE RELATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE AND CITRATE TO THE METABOLISM OF TESTOSTERONE BY LIVER TISSUE*

BY MAX L. SWEAT AND LEO T. SAMUELS

(From the Department of Biological Chemistry, University of Utah School of Medicine, Salt Lake City)

(Received for publication; February 24, 1948)

In a previous paper (1) from this laboratory, evidence was given indicating that the testosterone-destroying mechanism of liver involves an oxidative enzyme. The observation that certain enzyme inhibitors brought about only partial inhibition suggested that some substrate or carrier previously accumulated in the system beyond the points of inhibition was used in the testosterone destruction. In this paper evidence is given which strongly indicates that diphosphopyridine nucleotide (DPN) and citrate are involved in the destruction of testosterone by the liver. The end-products in the presence of added DPN are not the same as in the presence of added citrate. This agrees with the conclusions of Samuels and Pottner (2) that probably more than one step is involved in the destruction of testosterone by the liver. A number of other compounds associated with known metabolic systems have been tried, but have been ineffective.

EXPERIMENTAL

The tissue incubations and testosterone analysis were carried out according to the procedure of Samuels (3) and Samuels, McCaulay, and Sellers (1) with the exception that the incubation flasks were aerated with oxygen immediately after the addition of tissue mince. The testosterone was determined spectrophotometrically with the 240 m μ absorption band caused by the α,β -unsaturated ketone structure. The 17-ketosteroids were determined by the Callow modification of the Zimmermann reaction (4). The DPN, hexose diphosphate, and adenosine-3-phosphate were obtained from the Schwarz Laboratories, the adenosine-5-phosphate from the Ernst Bischoff Company. The oxalacetic acid was prepared from the sodium ethyl ester. The other cofactors were prepared as sodium salts from the corresponding acids. The buffer solution was the same as that used in previous studies (1). Most of the cofactors were added as sodium salts, the ionic balance of the buffer being maintained by leaving out a corresponding amount of sodium chloride. The buffer solution containing

* This investigation was supported by grants from the Donner Foundation, Inc., Ciba Pharmaceutical Products, Inc., and the National Cancer Institute.

the cofactor was used to wash the minced liver into the incubation flask. The flask was then filled with oxygen and placed in the incubation bath as rapidly as possible. Incubation was halted by boiling as in previous work and the analytical procedures then carried out.

Results

The various substances which have been tried, together with their effect on testosterone metabolism, are given in Table I. Of these substances,

TABLE I

Rate of Destruction of Testosterone in Presence of Various Cofactors

The results are expressed as micrograms per gm. per hr.

Cofactor	Concentration	Fed rats		Fasted rats	
		Rate with cofactor	Rate without cofactor	Rate with cofactor	Rate without cofactor
	<i>M</i>				
Succinate	0.05	107	105*		
Oxalate	0.005	103	125*	17	38
Adenosine-3-phosphate	0.001	115		41	61
Hexose diphosphate	0.005	143		39	61
Pyruvate	0.05	119	126*	49	51
Glutamate	0.05	119	125*		
Adenosine-5-phosphate	0.001	128	125*		
Calcium	0.001	143	127*		
DPN	0.001	209	111*	153	49
Citrate	0.001	269	111*	236	49
	<i>per cent</i>				
Glucose	0.1	125	120		

In all incubation flasks 200 γ of testosterone were introduced with approximately 0.7 gm. of liver mince and 25 cc. of buffer solution.

The final concentrations of ions in the buffer solution were as follows: KCl 0.0056 M, $MgCl_2$ 0.0021 M, NaCl 0.08 M, Na_2HPO_4 - NaH_2PO_4 buffer (pH 7.4) 0.04 M.

* The buffer in these flasks also contained 0.1 per cent glucose.

only DPN and citrate increased the rate of testosterone destruction significantly. There is no correlation between the effect of these substances on oxygen uptake and their effect on testosterone destruction. The concentrations of succinate used increased the oxygen uptake the most, but had little or no effect on the rate of testosterone metabolism.

As our preparation of DPN was only 60 per cent pure, the question arose whether some other ingredient could not be the causative factor in increasing testosterone metabolism. Adenosine-5-phosphate is one of the main contaminants of this preparation. The addition of this substance, as seen in Table I, had no effect.

There was a marked difference between the products formed in the presence of DPN and those in the presence of citrate. 17-Ketosteroids appeared to be the main products formed in the presence of DPN (Table II). The addition of citrate, on the other hand, did not give rise to these substances although the hormone was destroyed at a comparable rate. It would thus seem that the two compounds act in different ways on the metabolic process.

Destruction of testosterone was found to be decreased in the livers of rats fasted for 48 hours (Table II). Addition of DPN or citrate increased this rate beyond that of the livers of fed rats without such additions. Nicotinamide

TABLE II

Effect of Nicotinamide, DPN, and Citrate on Destruction of Testosterone by Liver Tissue of Fed and Fasted Rats

The results are means of duplicate incubations, micrograms per gm. per hour.

Cofactor		Rate with cofactor		Rate without cofactor		Difference due to cofactor	
		Testosterone destroyed	17-Ketosteroids formed*	Testosterone destroyed	17-Ketosteroids formed*	Testosterone destroyed	17-Ketosteroids formed*
Fasted 48 hrs.	Nicotinamide, 0.04 M	64		38		26	
	DPN, 0.001 M	197	112	49	0	148	112
	Citrate, 0.001 M	235	0	49	0	186	0
Fed	Nicotinamide, 0.04 M	228		125		103	
	DPN, 0.001 M	203	70	111	0	98	70
	Citrate, 0.001 M	269	0	111	0	158	0

In all incubation flasks 200 γ of testosterone were introduced with approximately 0.7 gm. of liver mince and 25 cc. of buffer solution.

* 17-Ketosteroids estimated as androsterone.

tinamide in 0.04 M concentration increased the rate in both types of liver, but as seen in Table II, the increase was proportionate to the rate of destruction with the buffer solution alone. The absolute increase in the livers from fasted rats was, therefore, much less. We are inclined to believe that these observations can be explained in terms of inhibition of DPN-destroying nucleotidases by nicotinamide as shown by Mann and Quastel (5) and Handler and Klein (6, 7). If so, the smaller absolute influence of nicotinamide on destruction of testosterone in the fasting liver would indicate that there was less DPN available.

DISCUSSION

DPN and citrate appear to be specifically utilized in the metabolism of testosterone. The products of metabolism when DPN is incubated with

the hormone, however, are distinctly different from those formed in the presence of citrate. Whereas the presence of DPN gives rise to products of a 17-ketosteroid nature, those obtained in the presence of citrate do not appear to fall into this chemical grouping.

The enhancing effect of DPN does not seem to be through its effect on increasing metabolism via the glycolytic and Krebs' cycle, as direct addition of several important members of these cycles did not increase the rate of metabolism of the hormone.

Citrate also appears to act directly. The evidence already cited in connection with DPN rules out a general effect through the Krebs' cycle. Its influence, however, might also be due to its effect on calcium ion. Swingle, Axelrod, and Elvehjem (8) showed that calcium ions increased the destruction of DPN, apparently through the nucleotidase system. However, calcium ion was ruled out as a primary factor both by the ineffectiveness of oxalate on testosterone metabolism and the small but insignificant acceleration in the destruction of the hormone produced by the addition of calcium ion. Citrate, therefore, must act in some yet unidentified way.

The observations of Samuels and Pottner (2) that rat and chick liver differed in the metabolic pathways of testosterone destruction is in accord with these present experiments. The addition of DPN to rat liver results in the formation of products regularly found in large amounts in the presence of chick liver. The latter may have relatively larger amounts of coenzyme in proportion to the metabolic systems leading to the formation of substances which are not 17-ketosteroids. Citrate would seem to accelerate a reaction similar to the non-17-ketosteroid phase. Whether DPN and citrate are involved in consecutive reactions or in two separate pathways, however, is not yet determined.

The difference in the rate of metabolism of testosterone between livers from fasting and fed animals when incubated with nicotinamide is good evidence that DPN is a factor in the normal metabolism of testosterone. As Mann and Quastel (5) and Handler and Klein (6, 7) have shown, nicotinamide and DPN are competitors for diphosphopyridine nucleotidase. By increasing the amount of nicotinamide, the DPN is thus protected for other systems. Further, the work of Handler and Klein (6, 7) on the effect of broken tissue cells in inactivating DPN may explain our previous inability to obtain active cell-free extracts (1).

SUMMARY

Evidence is presented that DPN and citrate are involved in the metabolism of testosterone by the liver. The products of the reaction in the presence of DPN are largely 17-ketosteroids, while those in the presence of citrate are not.

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THE SPECIFIC ESTERASE ACTIVITY OF CARBOXYPEPTIDASE

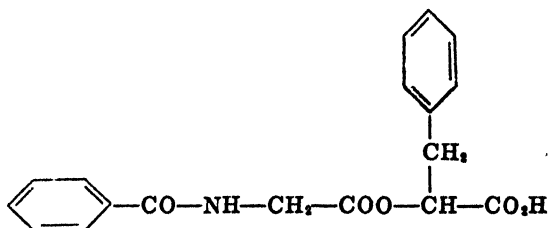
By JOHN E. SNOKE, GEORGE W. SCHWERT, AND HANS NEURATH

(From the Department of Biochemistry, Duke University School of Medicine,
Durham, North Carolina)

(Received for publication, March 11, 1948)

It was recently found that the proteolytic enzymes trypsin (1) and chymotrypsin (2) possess specific esterase activity. In addition to catalyzing the hydrolysis of specific peptides (3-7), these enzymes also catalyze the hydrolysis of those esters which possess the structural environment of the specific peptides. In order to substantiate further the suggestion of Schwert *et al.* (1) that the specific esterase activity is a general attribute of proteolytic enzymes, ester analogues of the specific peptide substrates for carboxypeptidase were prepared and the influence of the enzyme on these substrates was investigated. Two typical substrates for carboxypeptidase are carbobenzoxyglycyl-L-phenylalanine (8) and chloroacetyl-L-phenylalanine (8, 9). These substrates are enzymatically hydrolyzed to carbobenzoxyglycine and L-phenylalanine in the case of the former substrate, and to chloroacetate and L-phenylalanine in the case of the latter. If carboxypeptidase is endowed with specific esterase activity, the ester analogues of the above substrates, *i.e.* carbobenzoxyglycyl- β -phenyllactic acid and chloroacetyl- β -phenyllactic acid,¹ should likewise be hydrolyzed under the influence of carboxypeptidase.

The *carbobenzoxy* group of carbobenzoxyglycyl-L-phenylalanine is required, for it has been shown that carboxypeptidase will not act on substrates which contain a free amino group in close proximity to the susceptible peptide bond (8). However, it may be expected that some



¹ Preliminary measurements have been carried out on a preparation of chloroacetyl-*dl*- β -phenyllactic acid which resisted all attempts of crystallization and which, as an oil, gave a neutralization equivalent of 245 (theoretical 242.5). Hydrolysis by carboxypeptidase followed zero order reaction kinetics but failed to proceed beyond 17 per cent completion. For this reason, these results have to be considered to be tentative.

other substituent group which masks the basicity of the amino group will serve as well. For convenience, the *benzoyl* derivative of glycyl- β -phenyllactic acid was prepared (hippuryl- β -phenyllactic acid) instead of the corresponding carbobenzoxy derivative. As it has been shown (10) that the racemate of carbobenzoxyglycylphenylalanine is as suitable for kinetic studies as the L isomer, the substrate used in the present investigation was the racemic form of hippuryl- β -phenyllactic acid, the structure of which is given above.

This ester is readily hydrolyzed in the presence of carboxypeptidase. The results of quantitative kinetic studies are presented in this paper.

EXPERIMENTAL

Substrate, Hippuryl-dl- β -phenyllactic acid (HPLA)—The starting materials were hippuryl chloride and *dl*- β -phenyllactic acid. The preparation of hippuryl chloride was carried out as described by Fischer (11), while that of *dl*- β -phenyllactic acid was carried out according to the directions of Dakin and Dudley (12). The *dl*- β -phenyllactic acid was purified by recrystallization from hot water. A mixture of 5.3 gm. of hippuryl chloride, 4.5 gm. of *dl*- β -phenyllactic acid, and 50 cc. of dry toluene was refluxed for a period of 1 hour under anhydrous conditions. Upon being cooled, an orange gummy mass separated from the solvent which was then poured off. The reaction product was extracted with ether and the solvent was removed by concentration *in vacuo* to yield an orange oil. The oil was taken up in a hot ethanol-water mixture and treated with norit to yield a pale yellow solution. Upon concentration *in vacuo*, an oil resulted which crystallized on standing in the cold. The product was recrystallized from hot toluene, collected, and washed with a small amount of ether. Yield, 2.0 gm.; m.p., 121.5–122.5°.

$C_{18}H_{17}O_5N$ (327.3).	Calculated.	C 66.0, H 5.24, N 4.28
	Found.	" 65.9, " 5.21, " 4.38

Methods—Enzymatic measurements were carried out at 25° in the presence of 0.01 M phosphate buffer and 0.025 M LiCl. The substrate was weighed out before each experiment, and then neutralized to pH 7.5 by careful addition of 0.1 N NaOH. Buffer, LiCl solution, and water were added so that after the addition of the enzyme solution the total volume of the system would be 10 cc. The course of the reaction was followed by direct electrometric titration as previously described (1). Enzyme solutions were made up daily from a stock solution containing about 0.3 to 0.5 mg. of enzyme nitrogen per cc. The stock solution was prepared about every 3rd day from a stock suspension of crystals (10). Enzyme nitrogen concentrations were determined with the semimicro-Kjeldahl method.

Results

At pH 7.5, HPLA is rapidly hydrolyzed in the presence of small amounts of carboxypeptidase. Quantitative measurements revealed that the rate of hydrolysis follows zero order kinetics up to 80 per cent of the initial concentration of the enzymatically susceptible isomer of HPLA present in solutions of the *dl* substrate. Representative data for the hydrolysis of

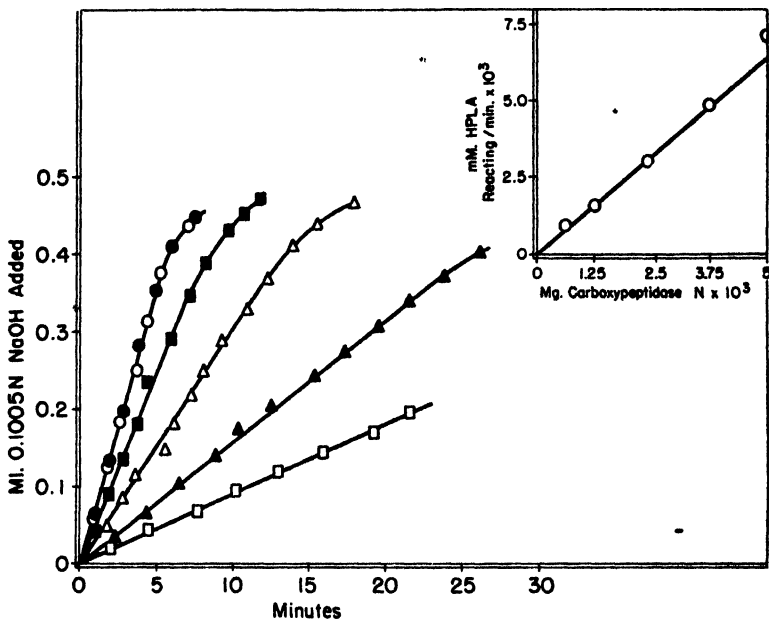


Fig. 1. Hydrolysis of HPLA by various concentrations of carboxypeptidase as measured by electrometric titration in 0.01 *M* phosphate buffer, 0.025 *M* LiCl, pH 7.50 at 25°. The initial concentration of *dl*-HPLA in all three systems was 0.010 *M*. The amount of carboxypeptidase *N* in mg. present in these systems (final volume 10 cc.) is shown by the following: ○ 0.00500, ● 0.00500, ■ 0.00375, △ 0.00250, ▲ 0.00125, □ 0.000625. The inset graph indicates the linear relationship between the rate of hydrolysis of HPLA and the amount of carboxypeptidase present.

0.01 *M* solution of *dl*-HPLA by five different concentrations of carboxypeptidase, varied over an 8-fold range, are given in Fig. 1. The precision of the analytical method is evidenced by the close fit of the points obtained in duplicate experiments at the highest enzyme concentration. The inset graph of Fig. 1 demonstrates that the rate of hydrolysis is proportional to the enzyme concentration in the system.

Although careful examination of the analytical data excluded rates other than that of zero order, a dependence of the rate constant on initial substrate concentration became apparent. Representative data are given in

Fig. 2, in which the titration data for four different initial substrate concentrations at a constant enzyme concentration of 2.5×10^{-4} mg. of N per cc. are plotted according to zero order kinetics. The rate of hydrolysis, represented by the slope of the linear portion of the curves, is highest for the lowest initial substrate concentration and decreases with increasing substrate concentration as shown in the inset graph of Fig. 2. The substrate concentration dependence of the reaction constant decreases mark-

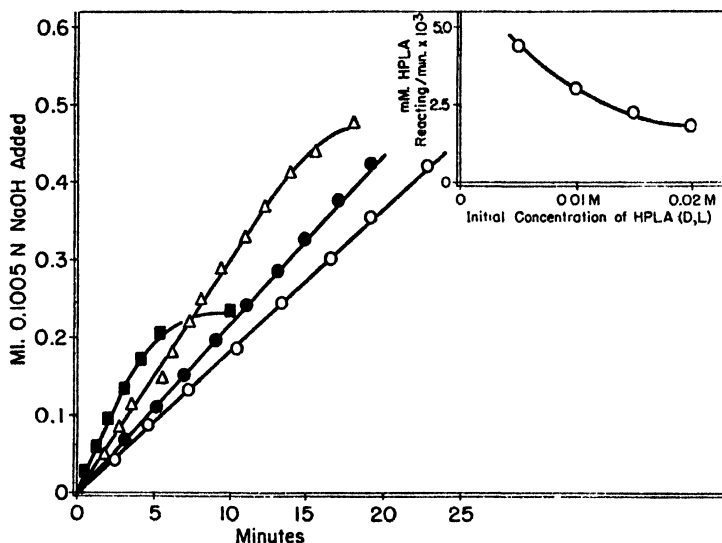


FIG. 2. Dependence of hydrolysis of HPLA on initial substrate concentration in 0.01 M phosphate buffer, 0.025 M LiCl, pH 7.50 at 25°. The amount of carboxypeptidase N in mg. present in these systems (final volume 10 cc.) was 0.0025. The initial concentration of *dl*-HPLA is shown by the following: ■ 0.005 M, △ 0.010 M, ● 0.015 M, ○ 0.020 M. The inset graph indicates the relationship between the rate of hydrolysis of HPLA and the initial concentration of HPLA.

edly with increasing substrate concentration and appears to vanish near 0.02 M *dl*-HPLA.

In view of the inhibition of carboxypeptidase activity toward carboxyglycyl-L-phenylalanine by chloroacetate (8, 9) and by D-phenylalanine (13), the influence of these two compounds as well as that of *dl*-phenyllactic acid on the hydrolysis of HPLA was investigated. No measurable effect was noted when 0.007 M sodium chloroacetate was added to the enzyme-substrate system containing 0.01 M *dl*-HPLA. The additions of 0.01 M DL-phenylalanine and of 0.01 M *dl*-phenyllactic acid to the same system decreased the zero rate of hydrolysis by, respectively, 38 and 17 per cent. A more detailed study of the inhibition by these two com-

pounds will be deferred until analogous experiments on their influence on the hydrolysis of carbobenzoxyglycyl-L-phenylalanine have been completed.²

The enzymatic specificity of the hydrolysis of HPLA was ascertained by the exclusion of a measurable extent of spontaneous hydrolysis of this ester in the buffer system used, and by measurements of the effects of other pancreatic proteolytic enzymes. Of the latter, crystalline ribonuclease³ (0.3 mg. per cc.) and crystalline trypsin⁴ (0.1 mg. of N per cc.) had no measurable effects. Crystalline chymotrypsin⁴ caused a slow hydrolysis, the rate per mg. of enzyme N per cc. being about 0.0005 of that produced by carboxypeptidase, *i.e.* 6.2×10^{-7} mole per minute per mg. of enzyme N for chymotrypsin compared to 1.2×10^{-3} for carboxypeptidase.

DISCUSSION

The present experimental data provide evidence that carboxypeptidase is capable of catalyzing the hydrolysis of a specific ester. The chemical structure of the substrate is such as to allow only one interpretation for the liberation of titratable acid during enzymatic hydrolysis, namely the splitting of the ester bond, since carboxypeptidase is incapable of hydrolyzing the secondary peptide bond of carbobenzoxyglycyl-L-phenylalanine (4).

The observed zero order kinetics for the enzymatic hydrolysis of HPLA is in accord with analogous data for the specific esterase activity of trypsin (1). This may be ascribed in both cases to full saturation of the enzyme by the substrate, the rate-determining step apparently being that of the activation on the enzyme surface. With both enzymes, too, the hydrolysis of the corresponding peptide apparently follows first order kinetics (4, 14). However, in the present case, the dependence of the rate of ester hydrolysis on initial substrate concentration requires consideration. It has been found that the apparent first order reaction constants for the hydrolysis of carbobenzoxyglycyl-DL-phenylalanine by carboxypeptidase (13) and for the hydrolysis of specific peptides and esters by chymotrypsin (2) decrease with increasing substrate concentration. This dependence of first order reaction constants upon substrate concentration can be accounted for by the shift of equilibrium between combined and free enzyme with changing substrate concentration. However, as would be expected, the initial reaction velocity increases with increasing substrate concentration.

The same explanation cannot apply to the observed decrease of the reaction constant of ester hydrolysis with increasing substrate concentration. Since the ester hydrolysis follows zero order kinetics, the initial velocity,

² Elkins-Kaufman, E., and Neurath, H., manuscript in preparation.

³ We are indebted to Dr. Lawrence L. Lachat, Armour and Company, Chicago, for the ribonuclease, crystallized from alcohol, used in this experiment.

⁴ The preparation was the same as that described in a previous publication (1).

as well as the rate constant, decreases with increasing substrate concentration. Nor can incomplete dissociation of HPLA or of the reaction products at the pH of the electrometric titrations (pH 7.5) account for this effect. Electrostatic interactions between enzymes and substrate may conceivably be considered and will be evaluated in further experiments.

Because of the difference in order of the reaction rates, strict comparison of the specific esterase and peptidase activities of carboxypeptidase is difficult. Further limitations of such a comparison arise from the large and oppositely directed dependence of the hydrolysis rates of peptide and ester upon substrate concentration.

The specific ester substrates for trypsin and chymotrypsin that have so far been tested are limited to those which contain a terminal methyl or ethyl group.⁵ Although these esters are structural analogues of specific amide substrates for trypsin and chymotrypsin, it is doubtful that amides of this type occur in native proteins. The hydrolysis of HPLA by carboxypeptidase is a more convincing proof for the inability of proteolytic enzymes to differentiate between peptide and ester bonds, since N-substituted glycyphenylalanine is a natural substrate for carboxypeptidase and HPLA a true analogue thereof.

This work has been supported by grants from the Rockefeller Foundation, from the National Institute of Health, the United States Public Health Service, and from the Duke University Research Council.

SUMMARY

Crystalline carboxypeptidase exhibits esterase activity toward an ester analogue of a specific peptide substrate. Hippuryl- β -phenyllactic acid (HPLA) is hydrolyzed at a fast rate according to zero order kinetics. Quantitative studies on HPLA, including the effects of enzyme and substrate concentrations, are described.

The synthesis of hippuryl-*dl*- β -phenyllactic acid is described.

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STUDIES ON THE HEAT INACTIVATION OF LYSINE IN SOY BEAN OIL MEAL*

BY ROBERT JOHN EVANS AND HELEN A. BUTTS

(From the Department of Agricultural Chemistry, Michigan State College, East Lansing)

(Received for publication, March 22, 1948)

The early literature demonstrating the loss in nutritive value of proteins resulting from heat treatment has been reviewed by McCollum, Orent-Keiles, and Day (1). Greaves and coworkers (2, 3) observed that lysine supplementation improved the nutritive value of heat-treated casein but not of raw casein for rats. Waisman and Elvehjem (4) made similar observations with autoclaved edestin, and Block *et al.* (5) with a cooked cake mixture. Block, Jones, and Gersdorff (6) isolated equal amounts of lysine from acid hydrolysates of heated and unheated casein. Zittle and Eldred (7), using a specific L-lysine decarboxylase to determine lysine, confirmed the results of Block *et al.* (6). Mitchell and Block (8) found no destruction of lysine to result from pelleting and exploding an oat-corn-rye mixture, a process which lowered the nutritive value of the proteins. Dry heat treatment of proteins appears to bind lysine in such a manner that it cannot be utilized by the animal. Acid hydrolysis, but not digestion *in vivo*, frees the lysine so that it can be utilized. This is supported by the observation of Seegers and Mattill (9) that, although heat treatment lowered the nutritive value of beef liver for rats, the acid-hydrolyzed liver supplemented with tryptophan supported growth comparable to that obtained with unheated liver.

Autoclaving soy bean oil meal at high temperatures or for long periods of time decreases the availability of the lysine for growing chicks (10, 11) and turkey poults (12). Part of this loss of availability is due to actual destruction of lysine as determined by microbiological assay on the acid-hydrolyzed soy bean oil meal (13, 14).

Two types of heat inactivation of lysine apparently take place, one a destruction of the lysine, and the other a binding of the lysine in some form such that it is not liberated by digestion *in vivo* or by enzyme hydrolysis *in vitro* but is liberated by acid hydrolysis. Evans and McGinnis (14) observed that, of the lysine not destroyed by autoclaving soy bean oil meal at 130° for 60 minutes, only 5 per cent was made available by enzyme digestion *in vitro*. It was the purpose of this investigation to study further the two types of heat inactivation of lysine occurring during the autoclaving of soy bean oil meal.

* Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal article No. 954 (new series).

EXPERIMENTAL

A preliminary experiment was conducted in which 20 gm. portions of solvent-extracted soy bean oil meal,¹ soy bean protein,² or sucrose³ were autoclaved for 4 hours at 15 pounds pressure both with and without additions of 0.1 gm. of L-lysine hydrochloride, 0.1 gm. of DL-methionine,⁴ and 0.05 gm. of L-cystine. A mixture of the three amino acids was also autoclaved without any other material.

10 gm. portions of soy bean oil meal,¹ soy bean protein,² sucrose, or soy bean protein (8 gm.) plus sucrose (2 gm.) were used in the experiment reported in this paper. Portions of soy bean oil meal and soy bean protein were autoclaved or heated in a drying oven for 4 hours at 121° both with and without additions of 0.50 gm. of DL-lysine hydrochloride.⁴ One portion of soy bean protein was autoclaved for 15 minutes. The samples of sucrose plus soy bean protein were autoclaved for 4 hours with and without the addition of 0.50 gm. of DL-lysine hydrochloride.

Lysine was determined on the above samples by microbiological assay with *Leuconostoc mesenteroides* with a medium similar to that described by Sauberlich and Baumann (15). Acid hydrolysates of the materials were prepared by autoclaving 1.0 gm. with 20 ml. of 20 per cent hydrochloric acid for 8 hours at 15 pounds pressure. *In vitro* enzyme (trypsin + erepsin) digests of the materials were prepared on 1.0 gm. samples, as described by Evans (16). When the sucrose and sucrose plus lysine samples were autoclaved they took up water, becoming syrupy. The samples were dissolved in water and diluted to a volume of 100 ml. 10 ml. aliquots were used for the acid hydrolysis of the samples and concentrated hydrochloric acid was added to give a solution of 20 per cent HCl. 10 ml. aliquots were also used for the enzyme digestion studies, the 10 ml. replacing an equal volume of water in the digestion mixture.

Results

No destruction of L-lysine hydrochloride took place when it was autoclaved for 4 hours with methionine and cystine in the preliminary experiment. Lysine autoclaved with soy bean oil meal was 67 per cent destroyed and 7 per cent converted to a form from which biologically active lysine was freed by acid hydrolysis but not by enzymic digestion *in vitro*. Lysine added to sucrose was completely destroyed by autoclaving, whereas

¹ Commercial solvent-extracted soy bean oil meal furnished by Professor J. A. Davidson of the Department of Poultry Husbandry.

² "Alpha" protein furnished by The Glidden Company, Chicago.

³ Commercial beet sugar.

⁴ DL-Methionine and DL-lysine monohydrochloride were furnished by The Dow Chemical Company, Midland, Michigan.

33 per cent of that added to soy bean protein was destroyed and 23 per cent bound in a form from which biologically active lysine was not freed by enzymic digestion *in vitro*.

The results of the principal experiment are presented in Tables I and II. Soy bean oil meal was changed by autoclaving from a golden colored material to a dark brown. 43 per cent of the lysine present in the meal was destroyed by autoclaving, and 61 per cent less lysine was liberated by enzymic digestion *in vitro* from the autoclaved than from the unautoclaved

TABLE I

Influence of Heat Treatment of Soy Bean Protein or Soy Bean Oil Meal on Inactivation of Lysine in Protein

10 gm. portions of the materials were autoclaved or heated in a drying oven at 121° for 4 hours for the heat treatment. Decreases in the lysine content of the soy bean materials as determined on the acid hydrolysate after heat treatment are considered to be due to destruction of lysine; those determined on the enzymic hydrolysate as due to inactivation, including destruction.

Soy bean product	Heat treatment	Acid hydrolysis		Enzyme hydrolysis	
		Lysine content	Lysine destroyed	Lysine content	Lysine in-activated
		per cent	per cent of total*	per cent	per cent of total†
Oil meal	None	2.69	0	1.28	0
	Autoclaved, 4 hrs.	1.53	43	0.50	61
	Dry heat, 4 hrs.	2.69	0	1.28	0
Protein	None	4.65	0	3.07	0
	Autoclaved, 15 min.	4.67	0	3.08	0
	“ 4 hrs.	4.52	3	2.14	30
	Dry heat, 4 hrs.	4.64	0	2.77	10
Protein + sucrose‡	Autoclaved, 4 hrs.	2.46§	47	0.50	84

* Total lysine is considered to be the lysine content of the unheated material determined after acid hydrolysis.

† Total lysine is considered to be the lysine content of the unheated material determined after enzyme hydrolysis.

‡ 8.0 gm. of soy bean protein and 2.0 gm. of sucrose.

§ Lysine content in the soy bean protein.

meal. Autoclaving destroyed 37 per cent of the added DL-lysine hydrochloride and converted another 22 per cent of it to a form from which biologically active lysine was freed by acid but not by enzymic digestion. Dry heat did not affect the lysine in the soy bean oil meal but destroyed 10 per cent and inactivated an additional 32 per cent of the added lysine. The color of the meal was little affected by the dry heat treatment.

The soy bean protein was converted by autoclaving from a white to a golden brown product. Little destruction of the lysine in the protein

occurred, but 30 per cent less lysine was liberated from the autoclaved protein than from the unautoclaved by enzymic digestion *in vitro*. Of the added lysine, 7 per cent was destroyed and 24 per cent more was inactivated by autoclaving. Dry heat had little effect on the soy bean protein or on the lysine added to it.

When sucrose was autoclaved with DL-lysine hydrochloride, a black syrupy mass was obtained, which on dissolving in water had the color of crude molasses. All of the added lysine was destroyed.

Autoclaving a mixture of 20 per cent sucrose and 80 per cent soy bean protein produced a chocolate-brown product darker in color than the autoclaved soy bean oil meal. 47 per cent of the soy bean lysine was destroyed, and 84 per cent less lysine was liberated from the autoclaved than from the unautoclaved soy bean protein by enzymic hydrolysis *in vitro*. 56 per cent of the added lysine was destroyed and 19 per cent more was inactivated.

DISCUSSION

Lysine is made biologically inactive in two different ways when a complex material such as soy bean oil meal is subjected to sufficiently drastic heat treatment. Part of the lysine is destroyed; that is, it is converted to a substance which is not biologically active for *Leuconostoc mesenteroides* after acid hydrolysis. Part of the lysine is converted to a form resistant to enzymic digestion *in vitro* but active for *Leuconostoc mesenteroides* after acid hydrolysis. Block *et al.* (5) postulated the latter reaction to be one between the free carboxyl group of the dicarboxylic amino acids and the ϵ -amino group of lysine to form a new peptide linkage resistant to enzymic but not to acid hydrolysis.

Further evidence for the two types of lysine inactivation is presented in the present paper. Destruction of lysine was apparently most influenced by the sucrose present. Autoclaving DL-lysine hydrochloride with sucrose resulted in complete destruction of the lysine. The addition of sucrose to soy bean protein to give a 20 per cent level of sucrose increased the destruction of the lysine (both that contained in the protein and that added as DL-lysine hydrochloride) from about 5 per cent to about 50 per cent. Sucrose is the principal carbohydrate in soy bean oil meal, comprising 6 per cent of the meal and 27 per cent of the total carbohydrates (17). Soy bean oil meal contains 22 per cent carbohydrates but no reducing sugars (17). Stevens and McGinnis (18) observed that autoclaving lysine with cerelese (primarily glucose) for 4 hours rendered it unavailable for chick growth. This was probably caused by the destruction of lysine.

When no carbohydrate was present, the principal result of autoclaving soy bean protein was to convert the lysine to a form that was not made

biologically active by enzyme digestion *in vitro* but was activated by acid hydrolysis. This could be caused by the mechanism postulated by Block *et al.* (5). Little destruction of lysine occurred in the absence of carbohydrate.

Dry heat had little or no effect on the availability of the lysine contained in soy bean oil meal or soy bean protein (10 per cent inactivated). Dry heat inactivated 42 per cent of the DL-lysine hydrochloride added to soy bean oil meal, of which 10 per cent was destroyed, but had little effect on that added to soy bean protein. Apparently carbohydrate or some other constituents of the soy bean oil meal increased the magnitude of dry heat

TABLE II

Influence of Heat Treatment of Lysine in Presence of Soy Bean Oil Meal or Its Constituents on Inactivation of Added Lysine

10 gm. portions of the soy bean oil meal, soy bean protein, or sucrose were used. Lysine was added at a level of 500 mg. of DL-lysine hydrochloride (250 mg. of L-lysine hydrochloride). The samples were heated as indicated for 4 hours at 121°.

Carrier	Treatment	Acid hydrolysis		Enzyme hydrolysis		Lysine inactivated but not destroyed
		Added lysine recovered	Lysine destroyed	Added lysine recovered	Lysine inactivated	
		mg.	per cent	mg.	per cent	per cent
Soy bean oil meal	Autoclaved	157	37	102	59	22
	Dry heat	224	10	146	42	32
" protein	Autoclaved	232	7	173	31	24
	Dry heat	238	4	235	5	1
Sucrose	Autoclaved	0	100	13	95	0
Soy bean protein + sucrose*	"	110	56	63	75	19

* 8.0 gm. of soy bean protein and 2.0 gm. of sucrose.

inactivation and destruction. The dry heat treatment used by other investigators was much more drastic than that employed here.

That free DL-lysine hydrochloride may be more readily inactivated or destroyed than the lysine combined in protein form is indicated by the data. Dry heat destroyed 10 per cent of the lysine added to soy bean oil meal and inactivated 32 per cent more but did not influence the lysine combined in the soy bean oil meal protein.

A comparison of the results of the preliminary experiment with the data presented in Table II indicates that the amount of destruction of added lysine depends to some extent on the relative amounts of added lysine and the soy bean material it is added to. A greater proportion of the added lysine was destroyed in the preliminary experiment in which the ratio of

L-lysine hydrochloride to soy bean product was 1:200 than in the experiment reported in Table II in which the ratio was 1:40.

SUMMARY

Two types of heat inactivation of lysine were observed when soy bean oil meal was autoclaved for 4 hours. Approximately 40 per cent of the lysine, both that present in the soy bean oil meal and that added as DL-lysine hydrochloride, was destroyed. 60 per cent less lysine was liberated by enzymic digestion *in vitro* from the autoclaved than from the unautoclaved meal. 20 per cent of the added lysine was converted to a form from which active lysine was freed by acid but not by enzyme hydrolysis *in vitro*.

Sucrose was apparently the cause of most of the lysine destruction which occurred when soy bean oil meal was autoclaved. Very little loss of lysine occurred when soy bean protein was autoclaved in the absence of this sugar, but approximately 25 per cent of the lysine was converted to a form from which biologically active lysine was liberated by acid but not by enzyme hydrolysis *in vitro*. The addition of 20 per cent sucrose to the soy bean protein resulted in a 50 per cent destruction of the lysine by autoclaving.

Dry heat treatment did not destroy or inactivate nearly as much lysine as autoclaving did at the same temperature for the same time.

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STUDIES ON CAROTENOID METABOLISM

IX. CONVERSION OF CAROTENE TO VITAMIN A IN THE HYPOTHYROID RAT*

By CATHERINE E. WIESE, JOHN W. MEHL, AND HARRY J. DEUEL, JR.

(From the Department of Biochemistry and Nutrition, University of Southern California
School of Medicine, Los Angeles)

(Received for publication, March 22, 1948)

In the past 20 years, considerable data have been reported concerning thyroid function and carotene metabolism. Von Noorden (1) in 1907 was the first to suggest that a carotenemia may be associated with certain metabolic disturbances. Kunde (2) noted the appearance of xerophthalmia in rabbits which had been thyroidectomized for 8 to 12 months. Fasold and Heidemann (3) reported that thyroidectomy in goats decreased the vitamin A content of the milk and increased the carotene content. That the thyroid is necessary for the conversion of carotene to vitamin A is maintained by Wendt (4), who noted that patients with Graves' disease had low serum vitamin A, even though their carotene intake was sufficient to produce a normal value. The subject has been reviewed rather completely by Drill (5) in 1943.

Recently, Canadell and Valdecasas (6) reported that carotene was unable to relieve the ocular symptoms of a vitamin A deficiency in a thiouracil-treated animal. However, these symptoms were alleviated if thyroid powder was administered along with carotene. Drill and Truant (7), using thyroidectomized animals, could not prevent or alleviate ocular symptoms by injecting 10 γ of carotene per day. To study the effect of the thyroid on carotenoid metabolism, Johnson and Baumann (8) used thiourea- and thiouracil-treated rats and found that after administering carotene very little vitamin A was stored in the liver and that thyroxine restored the ability of these animals to convert carotene to vitamin A. However, Remington *et al.* (9) reported that an oral dose of 0.6 γ of carotene per day was able to bring about a cure of the eye symptoms of vitamin A-deficient thyroidectomized rats within 7 to 9 days.

In a preliminary report by the authors (10) no significant effect of thyroxine on the conversion of carotene to vitamin A could be established. This was shown by administering carotene to thiouracil-treated vitamin A-

* These data are from a thesis presented by Catherine E. Wiese to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Master of Science.

Aided by a grant from the Nutrition Foundation.

TABLE I

Summary of Bioassays on Vitamin A-Deficient Rats Receiving Vitamin A in Cottonseed Oil or Cottonseed Oil Alone (Negative Controls) on Normal Vitamin A-Deficient Diet or on One Containing Thiouracil

The average results on males and females are weighted equally. When animals died during the course of the experiments, the number of animals still alive which are included in the average is given in parentheses.

Group No.	Vitamin A per day	No. of rats		Depletion period			Assay period								Thyroid weight per 100 gm. body weight
		Male	Female	Average weight at start	Average duration	Average final weight	Average increase in body weight up to (days)						Average final weight		
							5th	10th	15th	20th	25th	28th			
Regular vitamin A depletion diet															
1	1 U.	4	8	gm	days	gm	gm	gm	gm	gm.	gm.	gm.	gm	mg.	
2	2.50	3	9	42.3	22.4	89.6	-1.7	8.1	15.8	20.3	30.1	31.6	121.6	12.9	
3	10.0	4	8	43.1	25.7	94.3	6.8	19.9	30.9	41.2	46.8	50.5	144.8	13.1	
4	0.0	4	9	42.8	24.0	90.8	11.6	25.8	44.5	55.7	66.3	70.6	162.0	10.0	
										(11)	(11)				
										(11)	(11)				
										(12)	(10)	(6)	(3)	(1)	
Vitamin A depletion diet containing 0.5% thiouracil															
5	0.75	4	4	42.3	22.4	89.6	-1.7	8.1	15.8	20.3	30.1	31.6	121.6	12.9	
6	1.25	8	7	43.1	25.7	94.3	6.8	19.9	30.9	41.2	46.8	50.5	144.8	13.1	
7	2.50	7	7	42.8	24.0	90.8	11.6	25.8	44.5	55.7	66.3	70.6	162.0	10.0	
8	5.00	7	7	41.1	24.6	92.9	-3.7	-4.5	-9.2	-15.8	-21.3	-24	63.0		
9	10.0	7	7												
10	20.0	7	8												
11	0.0	7	8												
										</					

* Continued for a second 28 day period as Group 7a on 2.5 i.u. of vitamin A per day but given 6.5 γ of thyroxine per 100 gm. of body weight daily.

† Continued for a second 28 day period as Group 8a with the same dose of 5 i.u. of vitamin A daily.

‡ Continued for a second 28 day period as Group 10a with the vitamin A dose raised to 200 i.u. daily.

deficient rats and by measuring the resulting storage of vitamin A in the liver. However, there remained the possibility of impaired utilization of

vitamin A formed from carotene. The following experiment was designed to demonstrate the lowest level of carotene and vitamin A which would support growth in a thiouracil-treated animal and the level which would give maximum growth.

TABLE II

Summary of Bioassays on Vitamin A-Deficient Rats Receiving β -Carotene in Cottonseed Oil or Cottonseed Oil Alone (Negative Controls) on Normal Vitamin A-Deficient Diet or on One Containing Thiouracil

The average results on males and females are weighted equally. When animals died during the course of the experiments, the number of animals still alive which are included in the average is given in parentheses.

roup No.	Caro- tene per day	No. of rats		Depletion period			Assay period								Thyroid weight per 100 gm. body weight
		Malē	Female	Average weight at start	Average dura- tion	Average final weight	Average increase in body weight up to (days)						Aver- age final weight		
							5th	10th	15th	20th	25th	28th			
Regular vitamin A-deficient diet															
12	0.5	8	3	43.5	21.4	79.1	1.7	7.5	14.2	22.8 (10)	29.1 (10)	29.5 (10)	107.9		
13	1.0	6	5	44.1	21.3	82.8	6.5	18.6	29.8	43.4	52.3	55.7	138.3		
14	0.0	3	6	45.6	22.7	86.9	-1.6	-3.0	-6.1 (7)	-6.6 (5)	-13.8 (4)	-14.0 (3)	88.7	17.5	
Vitamin A depletion diet containing 0.5% thiouracil															
15	0.5	6	5	42.6	20.1	68.5	-1.2	-2.6	-3.9 (8)	-3.9 (7)	-4.7 (7)	-4.1 (7)	64.6	98.5	
16	1.0	6	4	42.8	22.0	68.1	-0.2	1.6	2.2	4.0	4.6	5.0	73.1		
17	2.0	6	3	43.1	21.7	68.9	1.8	2.2	3.0	3.8	5.7	6.4	75.3		
18	0.0	4	6	42.1	21.6	67.2	-2.5	-5.0	-7.3 (7)	-3.6 (5)	-7.2 (5)	-11.6 (5)	53.6	92.3	

Procedure and Results

Animals from our stock colony for use in the vitamin A bioassay were weaned and depleted of vitamin A according to the method prescribed in the United States Pharmacopoeia XII. Hypothyroid symptoms were produced in part of the group by the administration of 0.5 per cent thiouracil¹ in their diet from weaning. Thiouracil was continued in their diet

¹ We wish to thank the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, for their gift of thiouracil used in this experiment.

until the experiment was terminated. Animals were supplemented daily with levels of 0.5, 1.0, and 2.0 γ of carotene² dissolved in cottonseed oil or levels of 0.75, 1.25, 2.5, 5.0, 10.0, and 20.0 I.U. of vitamin A acetate³ dissolved in cottonseed oil. After 28 days on the assay, the vitamin A supplement in the group of thiouracil-treated rats receiving 20 I.U. daily was increased to 200 I.U. daily for another 28 days; those on 5.0 I.U. were continued as such for a second 28 day period; and those receiving 2.5 I.U. were injected daily with 6.5 γ of thyroxine per 100 gm. of body weight during a second 28 day interval. The thyroid glands of each rat were removed and weighed when the assay terminated. The data obtained in

TABLE III

Summary of Bioassays on Vitamin A-Deficient Rats Previously on 28 Day Bioassay Test, Receiving Vitamin A in Cottonseed Oil and (Group 7a) Also Thyroxine

The vitamin A depletion diet in the previous and current tests contains 0.5 per cent thiouracil.

The average results on males and females are weighted equally. When animals died during the course of the experiments, the number of animals still alive which are included in the average is given in parentheses.

Group No.	Vitamin A dose per day		Thyroxine administered daily	No. of rats		Average starting weight	Assay period							Thyroid weight per 100 gm. body weight
	First period	Present test		Male	Female		Average increase in body weight up to (days)						Average final weight	
							5th	10th	15th	20th	25th	28th		
	I.U.	I.U.	γ			gm.	gm.	gm.	gm.	gm.	gm.	gm.	gm.	mg
7a	2.50	2.50	6.5	7	4	81.5	8.5	17.5	28.5	41.5	51.4	55.1	136.6	19.8
8a	5.00	5.00	0.0	7	7	77.2	-0.9	-0.7	0.8	1.6	2.8	3.0	77.9	119.6
								(12)	(12)	(12)	(12)	(11)		
10a	20.0	200.0	0.0	6	7	79.2	-2.3	1.5	2.9	4.4	5.8	6.6	89.4	102.1
								(12)	(11)	(11)	(10)	(10)		

the growth tests are recorded in Tables I to III. The weight gains are plotted against the logarithm of the dose of vitamin A or carotene in Fig. 1.

DISCUSSION

The interpretation of the growth response to vitamin A or carotene is complicated by the effect of hypothyroidism on the growth of immature rats. The thiouracil-treated animals weigh less at the end of the depletion

² Crystalline carotene was obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

³ Obtained from Distillation Products, Inc., Rochester, New York

period than normal animals, and consequently lose less weight during the assay period when not receiving vitamin A. Likewise, when these thiouracil-treated animals are provided with an optimum supplement of vitamin A, their maximum gain in weight is less than that of normal animals. The gains in weight of immature normal and hypothyroid rats cannot, therefore, be made the direct basis for a comparison of the effect of vitamin A in the two groups. However, there may be some justification for comparing the amounts of vitamin A required to produce maximum growth in the two groups. A graphic comparison has been attempted in Fig. 1, A. It will be evident that 2.5 i.u. of vitamin A per day produce nearly maxi-

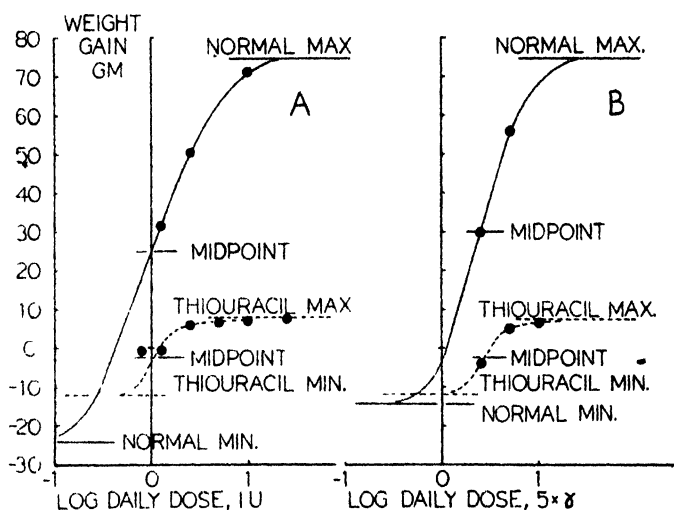


FIG. 1. The gain in weight of rats in gm. over a 28 day bioassay plotted against the log dose of vitamin A in international units (A) and against the log dose of β -carotene in micrograms (B) for normal rats (solid line) and for hypothyroid rats (dash line).

imum growth in both groups. The amount of vitamin A required to produce half of the maximum growth is close to 1 i.u. in both cases. There is a considerable uncertainty, of course, in the value to be taken for the weight loss of animals receiving no vitamin A, since a considerable number of these control animals die during the assay period. Nevertheless, it is difficult to arrive at any conclusion other than that vitamin A is about as effectively used for growth in the thiouracil-treated animal as in the normal animal, provided that the limitations imposed by hypothyroidism are considered. If the suggestion of Johnson and Baumann (8) regarding the importance of the size of the animal with respect to the retention of vitamin A is accepted, it may be argued that the utilization of vitamin A is less

effective in the thiouracil-treated animals. Since the thiouracil-treated animals weigh only about half as much as the normal animals at the end of the assay period, they would be expected to require only half as much vitamin A for maximum growth. This is clearly not the case.

The results of the experiments with carotene supplements are similar to those obtained with vitamin A. As indicated in Fig. 1, *B*, half of the maximum growth is obtained with a supplement of approximately 0.5 γ of β -carotene in both normal and thiouracil-treated animals. The relative effectiveness of vitamin A and β -carotene in promoting the growth of thiouracil-treated rats does not differ materially from that found in normal rats. In both cases, 0.4 to 0.6 γ of β -carotene produces the same weight response as 1 I.U. of vitamin A. Although it is impossible to conclude that the thiouracil-treated animals were entirely deficient in thyroid hormone, their limited growth and the degree of thyroid hypertrophy prove that they are severely hypothyroid. The thyroid hormone cannot be essential for the conversion of carotene to vitamin A, or it must be effective in very small amounts. Furthermore, if the thyroid hormone is concerned in the conversion of carotene to vitamin A, this process is not seriously impaired in severely hypothyroid rats.

The fact that growth of the thiouracil-treated rats is limited by the lack of thyroid hormone directly is demonstrated by two observations. In the first place, animals given a supplement of 200 I.U. of vitamin A per day during a second 28 day period comparable to the assay period, with thiouracil feeding, did not gain significantly more than animals continued on a supplement of 5 I.U. On the other hand, when the animals receiving a daily supplement of 2.5 I.U. of vitamin A and thiouracil in the diet were continued on the same regimen for a second 28 day period, but with 6.5 γ of thyroxine per 100 gm. of body weight daily (Group 7a), the average weight gain was 55.1 gm. This may be compared with the weight gain of 50.5 gm. for the normal animals supplemented with 2.5 I.U. of vitamin A during the regular assay period.

Although the vitamin A required for growth does not seem to be altered in the thiouracil-treated animals, the survival time of the negative control groups was considerably prolonged in the thiouracil-treated animals. In the group used in the vitamin A tests (Table I), the average survival time of the negative control animals receiving no thiouracil was 18.1 days, while that of the negative controls receiving thiouracil was 25.7 days. These values represent minimum differences, since a survival time of 28 days is assigned to those rats still alive at the end of the test. Only one (8 per cent) of the rats in the negative control group survived the 28 day period, while ten (67 per cent) of the rats receiving thiouracil in addition to the vitamin A-free diet were still alive after 28 days. The differences

are similar but less striking in the negative controls used in the carotene series.

This same general conclusion may be arrived at by considering the development of xerophthalmia. Eye symptoms developed in 100 per cent of the control group which did not receive thiouracil, while eye symptoms were observed in 60 per cent of the thiouracil-treated control group. These results, together with those on survival, might indicate that the small stores of vitamin A remaining at the end of the depletion period are utilized more slowly in the thiouracil-treated rat. Such an increased efficiency in utilization of vitamin A is not indicated in the growth experiments. The converse of this situation has been reported by Ershoff and Deuel (11), who found that the survival time of vitamin A-deficient rats was markedly shortened when a preparation of growth hormone was injected. Since these latter animals did not grow, being deficient in vitamin A, the stress which resulted in shorter survival times cannot be growth *per se*. It would seem that the increased demand for vitamin A must be due to a different factor from the requirement for growth in either case.

It is difficult to reconcile these results with those of Johnson and Baumann (8) on liver storage, and they would be in better agreement with the result previously obtained in this laboratory (10). However, more recent experiments in this laboratory have suggested that thiouracil treatment does decrease the amount of vitamin A stored in the liver of rats as the result of feeding carotene. If Johnson and Baumann are correct, then it would be necessary to conclude that the determination of the amount of vitamin A stored in the liver after feeding relatively large amounts of carotene is a more sensitive test of the ability to convert carotene to vitamin A than a bioassay procedure as applied in this case.

SUMMARY

1. Animals rendered hypothyroid by the inclusion of thiouracil in the diet responded to vitamin A and carotene administration by increased growth and by alleviation of the ocular symptoms.

2. Although the extent of maximum growth after vitamin A or carotene feeding is markedly depressed by hypothyroidism, the point of 50 per cent response was unaltered either with the vitamin A or carotene feeding. This was approximately 1 I.U. for vitamin A and 0.50 γ for carotene. These data indicate that carotene and vitamin A are about equally well utilized by hypothyroid rats at low levels of vitamin A or carotene.

3. When the rate of growth is limited by thiouracil feeding, it can be restored to normal by thyroxine but not by large doses of vitamin A.

4. Survival time of rats on a vitamin A-deficient diet is much prolonged

when the animals are rendered hypothyroid by thiouracil feeding. This might indicate that the stored vitamin A is used up at a slower rate under such conditions and hence may be available over a considerably longer period or that the factors on which the requirement for vitamin A is based differ in respect to growth and survival.

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EXCRETION OF AMINO ACIDS BY RATS AND MICE FED PROTEINS OF DIFFERENT BIOLOGICAL VALUES*

BY H. E. SAUBERLICH, E. L. PEARCE, AND C. A. BAUMANN

(From the Department of Biochemistry, College of Agriculture,
University of Wisconsin, Madison)

(Received for publication, April 3, 1948)

In a previous study it was observed that mice fed diets devoid of certain essential amino acids excreted very high percentages of all the ingested amino acids into the urine and that more amino acids were excreted when a relatively poor protein such as arachin was fed than when the dietary protein was casein (1). In the present study the proteins fed were known to be of higher biological value for the rat than is casein (2). Furthermore, a preliminary attempt was made to determine whether the excretion of amino acids by the rat depends upon the quality of protein ingested.

Methods

In the first series, weanling mice averaging 13.3 gm. in weight were divided into five comparable groups of four each and were fed diets of the following composition:

	per cent
Protein	8
Corn oil (+ 0.1 % halibut liver oil)	5
Wesson's salt mixture (3)	4
Glucose monohydrate (cerelose) to	100
	γ per gm.
Pyridoxine hydrochloride	6
Thiamine chloride	6
Nicotinic acid	10
Calcium pantothenate	20
Riboflavin	6
Biotin	0.5
p-Aminobenzoic acid	300
Inositol	500
Choline chloride	1000

In the second series the percentage of dietary protein was increased to 10 per cent. The proteins fed in both series included casein, egg albumin,

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and the American Cancer Society.

lactalbumin, fibrin, and arachin. The various proteins were air-dried before incorporation into the diet fed. Samples of these proteins were hydrolyzed (1) and analyzed for their amino acid composition by the same methods that were used for the determination of the amino acids in the urine. The composition of the proteins fed is summarized in Table I. The composition of the oxidized casein has been reported previously (1).

In both series each mouse received 1 drop of halibut liver oil every 2 weeks. The experiments were conducted for 7 weeks; urine from groups of four mice was collected under toluene for 48 hours at weekly intervals

TABLE I
Amino Acids in Proteins Fed

The results are expressed in gm. of amino acid liberated per 100 gm. of air-dried protein.

Amino acid	Lactalbumin	Fibrin	Arachin	Casein	Egg albumin	Zein
Arginine	3.3	6.8	11.0	3.7	5.3	2.0
Aspartic acid ..	10.3	15.5	14.0	7.3	8.4	6.6
Cystine	2.5	1.8	0.9	0.45	3.9	1.1
Glycine	2.9	4.9	3.2	1.8	3.0	0.4
Glutamic acid..	14.1	13.0	21.6	21.5	9.5	27.0
Histidine....	2.1	2.5	2.6	3.0	2.5	1.7
Isoleucine ..	7.8	7.6	6.6	7.1	6.9	7.3
Leucine.....	11.3	6.7	6.9	10.5	7.1	23.7
Lysine	7.1	6.8	1.8	7.7	5.7	0.0
Methionine	2.4	2.6	1.1	3.1	3.0	2.2
Phenylalanine..	3.4	3.8	5.5	5.1	4.9	7.4
Proline.	3.4	4.6	6.0	13.1	7.1	15.0
Serine	6.8	11.2	9.9	7.0	8.3	8.3
Threonine ..	4.0	5.9	2.6	4.6	4.1	2.4
Tryptophan	2.2	3.6	0.69	1.4	1.4	Trace
Tyrosine ..	3.1	4.6	4.5	5.4	3.8	6.0
Valine	4.7	3.7	3.9	6.3	3.6	2.4

for three weeks and twice more thereafter. The samples were filtered^a adjusted to pH 6.8, diluted to an equivalent of 25 ml. per mouse per day, and stored in the refrigerator under toluene for the microbiological determination of sixteen amino acids by the methods previously described (4). In addition glycine was determined with *Leuconostoc mesenteroides* P-60 with Medium III (4).

The rats were of the Sprague-Dawley strain and weighed 95 to 105 gm. They were fed diets of the following composition:

	per cent
Protein	12
Corn oil (+ 0.1 % halibut liver oil)	5

	per cent
Weesson's salt mixture (3).....	4
Glucose monohydrate (cerelose) to	100
	γ per gm.
Pyridoxine hydrochloride	6
Thiamine chloride.....	6
Nicotinic acid.....	10
Calcium pantothenate	20
Riboflavin.....	6
Biotin	0.5
Folic acid.....	0.5
p-Aminobenzoic acid	800
Inositol	500
Choline chloride...	1000
L-Cystine.....	1000

The proteins fed were ordinary casein or oxidized casein (5) with supplements in special groups of 0.4 per cent DL-tryptophan or 0.8 per cent DL-methionine or both. The rats were maintained on these diets for 4 weeks. In a second series adult rats averaging 200 gm. in weight were maintained on similar diets for 7 weeks. After 21 days on the diets the rats were placed in metabolism cages, singly or in pairs, and the urine was collected under toluene for 1 or more days. The samples were then filtered, adjusted to pH 6.8, and diluted with water to an equivalent of approximately 75 ml. per rat per day. The diluted samples were then stored in the refrigerator under toluene for the microbiological determination (4) of five representative amino acids: glutamic acid, histidine, lysine, phenylalanine, and valine. Aliquots of the urine samples were hydrolyzed with 2 N HCl in the autoclave for 6 hours at 15 pounds pressure. The samples were then neutralized with NaOH, made to volume, filtered, and stored in the refrigerator under toluene.

EXPERIMENTAL

Amino Acid Excretion by Mice Fed Various Proteins—The mice fed the various proteins at 8 per cent of the diet grew at rates that varied with the nature of the protein fed. Growth was poorest when the protein was arachin, on which the gains in weight averaged only 0.6 gm. in 4 weeks in the series fed 8 per cent of protein (Table II) as contrasted with an average gain of 6.7 gm. during this time when casein was fed. Egg albumin, lactalbumin, and fibrin promoted better growth than casein, with gains of 7.6 to 10.0 gm. in 4 weeks (Table II). Similar differences between proteins were noted when the diet contained 10 per cent of protein.

The amounts of the amino acids ingested and excreted on a typical collection date, the 14th day of the first series, are presented in Tables III and IV. A summary of the mean percentages of the ingested amino acids

excreted during the entire experimental period is presented in Table V. No attempt is made to present all of the results of the many analyses completed. In general, however, the percentages of the various amino

TABLE II
Effect of Various Proteins at 8 Per Cent Levels upon Growth of Young Mice

Diet	Average weight per mouse (4 mice per group)				
	0 wk.	1 wk.	2 wks.	3 wks.	4 wks.
	gm.	gm.	gm.	gm.	gm.
Arachin	13.8	13.0	14.4	14.1	14.4
Casein	13.2	14.2	16.8	18.2	19.9
Egg albumin	13.2	16.3	17.8	18.5	20.8
Fibrin	13.4	13.0	17.2	19.0	23.2
Lactalbumin	13.2	16.2	20.1	20.5	23.2

TABLE III
Microbiologically Available "Free" Amino Acids in Urine of Mice Fed 8 Per Cent Casein or Egg Albumin Diet for 14 Days

Amino acid	Ordinary casein			Egg albumin		
	Amino acid ingested	Amino acid excreted	Ingested amino acid excreted	Amino acid ingested	Amino acid excreted	Ingested amino acid excreted
	mg.	mg.	per cent	mg.	mg.	per cent
Arginine	8.91	0.60	6.7	10.78	0.25	2.3
Aspartic acid	17.60	0.27	1.5	17.09	0.03	0.2
Glutamic "	51.77	1.13	2.2	19.30	0.45	2.3
Histidine	7.23	0.32	4.4	5.08	0.05	1.0
Isoleucine	20.69	0.61	2.9	14.02	0.21	1.5
Leucine	25.28	1.13	4.5	14.43	0.28	1.9
Lysine	16.12	0.58	3.6	11.58	0.13	1.1
Methionine	7.47	0.17	2.3	6.10	0.12	2.0
Phenylalanine	11.56	0.34	2.9	9.96	0.11	1.1
Proline	38.25	2.75	7.2	14.43	0.40	2.8
Serine	19.00	0.40	2.1	16.85	0.17	1.0
Threonine	11.08	0.38	3.4	8.33	0.20	2.4
Tryptophan	3.37	0.09	2.7	2.85	0.03	1.1
Tyrosine	12.52	0.43	3.4	7.72	0.12	1.6
Valine	14.37	0.69	4.8	7.32	0.30	4.1
Mean for all acids determined			3.4			1.6

acids excreted on any particular protein were very similar whether the level of protein in the diet was 8 per cent or 10 per cent. On any given protein, the variations between the percentage excretion of a single amino

acid on different days during the experiment were frequently greater than the variations between the percentages of excretion for different amino acids on any one day. Thus, the percentages of tryptophan excreted by mice fed 8 per cent of casein were 3.4, 2.7, 2.1, 3.8, and 4.8 at 7, 14, 20, 24, and 40 days, respectively. By way of contrast, the percentages of nine different amino acids excreted on this diet on the 14th day of the experiment lay between the comparatively narrow range of 2.1 and 3.6 per cent (Table III).

TABLE IV

Microbiologically Available "Free" Amino Acids in Urine of Mice Fed 8 Per Cent Fibrin or Lactalbumin Diet for 14 Days

Amino acid	Lactalbumin			Fibrin		
	Amino acid ingested	Amino acid excreted	Ingested amino acid excreted	Amino acid ingested	Amino acid excreted	Ingested amino acid excreted
	mg.	mg.	per cent	mg.	mg.	per cent
Arginine	8.83	0.27	3.1	24.79	0.33	1.3
Aspartic acid	27.41	0.12	0.4	56.60	0.75	1.3
Glutamic "	37.68	0.52	1.4	47.42	1.07	2.3
Histidine	5.61	0.05	0.9	9.12	0.29	3.2
Isoleucine	20.84	0.28	1.3	27.69	0.75	2.7
Leucine	30.19	0.37	1.2	24.44	0.73	3.0
Lysine	18.97	0.19	1.0	24.80	0.71	2.9
Methionine	6.41	0.11	1.7	11.86	0.29	2.4
Phenylalanine	9.08	0.13	1.4	13.83	0.38	2.7
Proline	9.08	0.50	5.5	16.77	0.61	3.6
Serine	18.18	0.28	1.5	32.53	0.31	1.0
Threonine	10.69	0.22	2.1	21.52	0.71	3.3
Tryptophan	5.88	0.05	0.9			
Tyrosine	8.28	0.15	1.8	16.80	0.13	0.8
Valine....	12.54	0.30	2.4	13.47	0.73	5.4
Mean for all acids determined			1.4			2.7

The results indicated clearly, however, that the excretion of amino acids throughout the experiment was least on lactalbumin and egg albumin, intermediate on fibrin and casein, and highest on arachin. The mean percentages of excretion for all of the amino acids were 4.7, 3.4, 2.7, 1.5, and 1.0 on arachin, casein, fibrin, egg albumin, and lactalbumin, respectively (Table V). In other words, those proteins which promoted the most rapid rate of growth in the mouse were metabolized with the least losses of microbiologically available amino acids in the urine; the losses were significantly less on lactalbumin, egg albumin, and fibrin than on even such

a relatively good protein as casein. Arachin, on the other hand, enabled the mice to grow rather poorly and the excretion of amino acids when this protein was fed was significantly greater than that on the better proteins. When the better proteins were fed, no significant changes in the excretion of amino acids were noted as the experiment was prolonged; on arachin, however, the amino acid excretion increased after the 3rd week.

TABLE V

*Excretion of Microbiologically Available "Free" Amino Acids in Urine of Mice Fed Various Proteins in Diet at Level of 8 Per Cent**

Amino acid	Mean excretion of ingested amino acids				
	Arachin	Casein	Fibrin	Egg albumin	Lactalbumin
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Arginine	4.7	2.4	1.3	2.0	2.7
Aspartic acid . . .	1.9	1.5	1.3	0.2	0.2
Cystine	17.4	26.0	3.4	1.9	3.8
Glycine	11.5	14.0	7.6	8.0	6.2
Glutamic acid . . .	1.5	2.2	2.3	1.9	1.4
Histidine	5.0	4.4	3.2	1.0	0.9
Isoleucine	4.7	2.9	1.5	0.9	0.8
Leucine	3.3	3.7	3.0	1.5	0.7
Lysine	4.5	3.6	2.9	0.6	0.8
Methionine	4.1	2.5	2.7	1.5	1.7
Phenylalanine . . .	2.6	2.9	2.7	0.8	0.7
Proline	8.6	7.2	3.0	2.1	5.0
Serine	1.5	2.1	2.0	1.2	1.5
Threonine	6.0	3.8	4.1	2.4	1.5
Tryptophan	6.5	3.4	0.5	1.1	0.7
Tyrosine	4.7	3.6	0.4	0.8	0.7
Valine	8.2	4.8	0.8	3.4	1.0
Mean for all acids determined	4.7	3.4	2.7	1.5	1.0

* Average of five determinations on samples collected at intervals over a 40 day feeding period.

Amino Acid Excretion by Rats Fed Deficient Proteins—Rats fed oxidized casein as the sole source of amino acids or the oxidized casein supplemented with either tryptophan or methionine lost about 10 gm. per week throughout the experiment (Table VI), whereas those fed the oxidized casein supplemented with both tryptophan and methionine lost weight at a rate of 0.5 gm. per week for 4 weeks, as compared to a gain in weight of 10.2 gm. per week when ordinary casein was fed. In line with the experience of others, we found that the rats deprived of one or more essential amino acids ate less than those on the relatively complete diets, the average daily

food consumption being 4.6, 4.1, and 4.6 gm. during the 4th week, as compared to 8.4 gm. on the oxidized casein supplemented with tryptophan

TABLE VI

Amino Acids in Urine of Rats Fed Diets Low in Methionine or Tryptophan
12 per cent oxidized casein or ordinary casein was used.

Amino acid		Group I, oxidized casein	Group II, oxidized casein + trypto- phan*	Group III, oxidized casein + methio- nine†	Group IV, oxidized casein + methio- nine† + trypto- phan*	Group V, regular casein, <i>ad libitum</i>	Group VI, regular casein, restricted
Mg. of amino acid excreted daily							
Glutamic acid	"Free"	5.89	4.31	8.16	6.72	1.54	0.37
	Total	7.97	6.03	9.97	7.91	3.09	1.04
Histidine	"Free"	0.72	0.75	1.00	0.98	0.63	0.08
	Total	0.90	1.56	1.06	1.21	0.75	0.12
Lysine	"Free"	1.12	1.26	1.56	0.45	1.17	0.24
	Total	2.17	2.01	2.24	1.76	1.93	0.28
Phenylalanine	"Free"	1.29	1.23	1.25	1.53	1.02	0.10
	Total	1.45	1.27	1.33	1.71	1.02	0.13
Valine	"Free"	1.42	1.40	1.31	2.35	1.17	0.28
	Total	3.55	3.59	2.70	4.41	3.51	0.68
% of ingested amino acids excreted							
Glutamic acid	"Free"	4.89	4.01	6.75	3.05	0.62	0.24
	Total	6.62	5.62	8.24	3.59	1.25	0.67
Histidine	"Free"	5.44	6.35	7.58	4.10	1.84	0.38
	Total	6.82	13.28	8.01	5.02	2.19	0.55
Lysine	"Free"	2.73	3.44	3.76	0.59	1.34	0.44
	Total	5.27	5.50	5.40	2.31	2.22	0.51
Phenylalanine	"Free"	4.69	5.02	4.54	3.04	1.85	0.29
	Total	5.28	5.28	4.80	3.42	1.85	0.36
Valine	"Free"	4.11	4.56	3.76	3.72	1.61	0.62
	Total	10.29	11.70	7.77	6.98	4.85	1.50

Food eaten daily (average
throughout experiment),
gm.

Average gains in weight per
wk., gm.

* 0.4 per cent DL-tryptophan added to the diet.

† 0.8 per cent DL-methionine added to the diet.

and methionine and 9.6 gm. on ordinary casein. Rats fed the diet containing ordinary casein, but restricted to the amount consumed by the rats deprived of methionine, lost 2.0 gm. per week for 4 weeks.

The excretion of amino acids by the rats, as indicated by the amounts of glutamic acid, histidine, lysine, phenylalanine, and valine in the urine, depended both upon the amount and the quality of the protein fed. In spite of their relatively low intakes of food, the rats fed the deficient proteins (Table VI, Groups I, II, and III) usually excreted at least as much amino acid as those fed the more complete proteins (Table VI, Groups IV and V). The amounts of the amino acids excreted were usually less when ordinary casein was fed than on a diet of oxidized casein supplemented with tryptophan and methionine, and this difference became even more evident when the excretion of amino acids was expressed as the percentage of ingested amino acids appearing in the urine (Table VI). Presumably, therefore, the mixture of oxidized casein plus methionine and tryptophan was still somewhat inadequate as a source of protein for the rat. Since the excretion of "free" or microbiologically available lysine was particularly low on this diet (Table VI), it is possible that lysine may have been the limiting amino acid in the mixture fed.

When the food intake of the rats on ordinary casein was restricted (Table VI, Group VI) to that consumed by Group II fed the diet deficient in methionine, the excretion of "free" amino acids fell to very low levels, a mean of 0.5 per cent of the amino acids ingested (0.24 to 0.62 per cent) as compared to 5.5 per cent (4.0 to 13.28 per cent) by rats on the deficient diet, and 1.61 per cent (0.62 to 1.85 per cent) on ordinary casein fed *ad libitum* (Table VI, Groups VI, II, and V). Thus the rats on the deficient diets excreted about 10 times as much of the ingested amino acids in the "free" form as rats fed comparable amounts of a diet containing an adequate protein.

Determinations of the amino acids in hydrolyzed urine indicated that the effects of the various diets upon the excretion of total amino acids ("free" acids plus peptides) were very similar to their effects upon the excretion of the free acids; the highest percentages of ingested amino acids excreted were observed on the diets containing the incomplete proteins; an intermediate percentage was observed on ordinary casein, whereas the excretion of amino acids was particularly low when the latter diet was fed in the limited amounts consumed by the groups fed the deficient proteins. The various amino acids differed somewhat in their distribution between the "free" and the "bound" forms in urine: the amounts of phenylalanine excreted in the "free" form practically equaled those measured after hydrolysis (Table VI); in other words, little or no phenylalanine occurred in urine in the "bound" form. On the other hand, over half of the total amount of valine present became microbiologically available only upon hydrolysis, whereas the percentages of glutamic acid, histidine, and lysine excreted in the "bound" form were intermediate between valine and phenyl-

alanine. In general, the increases in amino acid excretion observed when deficient proteins were fed were found in both the "free" and the "bound" forms (Table VI). Determinations of α -NH₂-N in the urine samples by the copper method of Pope and Stevens (6, 7) tended to confirm the results of the microbiological assays of the individual amino acids.

DISCUSSION

The low excretion of amino acids by mice fed proteins of high biological value suggests that there is a rough inverse correlation between the growth-stimulating power of a protein and the percentages of ingested amino acids excreted in the urine. At low levels of dietary protein, casein was markedly inferior to lactalbumin in promoting growth, and higher percentages of all amino acids were excreted. Indeed in both respects casein was almost as inferior to lactalbumin as oxidized casein is to casein itself. The results suggest that amino acid excretion by the mouse might provide a fair index of the biological value of the protein mixture ingested.

The present experiments indicate that the rat resembles the mouse qualitatively in that it excretes more amino acids when the dietary protein is deficient in an essential amino acid than when the protein is biologically adequate. Schweigert has reached a similar conclusion (8). This loss in amino acids apparently is associated with a negative nitrogen balance (9). Quantitatively, however, the urinary losses of amino acids by the deficient rat are less spectacular than the amounts lost by deficient mice; such losses by mice averaged 24.5 per cent of the amounts ingested in a previous study (1). Another difference between the rat and the mouse may be the form in which extra amino acids are excreted when inadequate proteins are fed: the rat appears to excrete the excess acids in both the "free" and "bound" form, whereas the mouse excretes them largely in the microbiologically available form, either as free acids or as peptides that are directly useful to the assay organisms. For reasons discussed previously (1, 4), however, the distinction between "free" and "bound" amino acids in urine must be regarded as only approximate.

SUMMARY

1. Mice fed diets containing protein of high biological value excreted less "free" amino acids in the urine than mice fed proteins of ordinary or poor biological value. The mean excretion for seventeen amino acids determined by microbiological procedures was 4.7 per cent for arachin, 3.4 per cent for casein, 2.7 per cent for fibrin, 1.5 per cent for egg albumin, and 1.0 per cent for lactalbumin.

2. Rats fed restricted amounts of a diet containing ordinary casein excreted very low percentages of the ingested amino acids into the urine.

3. Rats fed diets containing incomplete proteins (oxidized casein plus cystine and either tryptophan or methionine) excreted approximately twice the percentage of ingested amino acids into the urine as those excreted by rats fed an adequate protein. The increased acids excreted were in both the "free" and the "bound" forms.

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ACTION OF CARBOXYPEPTIDASE ON PEPTIDE DERIVATIVES OF L-TRYPTOPHAN*

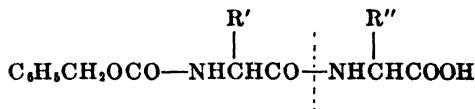
BY EMIL L. SMITH

(From the Laboratory for the Study of Hereditary and Metabolic Disorders, and the
Departments of Biological Chemistry and Medicine, University of Utah
School of Medicine, Salt Lake City)

(Received for publication, April 9, 1948)

It is obviously desirable to extend our knowledge of the specificity of proteolytic enzymes to as wide a variety of substances as possible. Thus far, the carbobenzoxy method of Bergmann and Zervas (1) has not been applied to the synthesis of compounds containing L-tryptophan. In fact, with the exception of the early work of Abderhalden and Kempe (2), and a recent report by Fruton (3), synthetic studies involving tryptophan peptides have not been reported. This paper will deal with the synthesis of a number of carbobenzoxy derivatives of tryptophan and its peptides, and the application of these compounds to the study of the specificity of crystalline pancreatic carboxypeptidase. Subsequent investigations will be concerned with tissue studies and with other enzymes.

From previous work (4-6), it is known that pancreatic carboxypeptidase can hydrolyze a wide variety of acylated peptides, but the rate of hydrolysis varies widely, being governed by the nature of the R groups present.



The dotted line shows the point of cleavage. The most sensitive substrates for this enzyme are those containing as the terminal amino acid L-phenylalanine or L-tyrosine. Since these compounds contain aromatic rings, it was of some interest to test the analogous L-tryptophan compounds. Our substrates were carbobenzoxyptides of the type indicated.

Carbobenzoxyglycyl-L-tryptophan (CGT) is rapidly hydrolyzed by carboxypeptidase. It is striking that the hydrolysis of this compound follows the kinetics of a zero order reaction over a wide range of enzyme concentrations (Table I), while the hydrolysis of other carbobenzoxyglycylamino acids at the same substrate concentration (0.05 M) is first order (4-6).

Table II shows that other tryptophan-containing peptides are also hydrolyzed by carboxypeptidase. Carbobenzoxy-L-tryptophyl-L-tryptophan

* This investigation was supported by a grant from the United States Public Health Service.

and carbobenzoxy-L-tryptophyl-L-tyrosine are split quite rapidly. However, since these compounds are sparingly soluble at pH 7.5, comparative kinetic studies could not be performed. The hydrolysis of carbobenzoxy-L-tryptophylglycine (CTG) and carbobenzoxy-L-tryptophyl-L-alanine (CTA) is best described as following the kinetics of a zero order reaction.

TABLE I

Kinetics of Carboxypeptidase Action of Carbobenzoxyglycyl-L-tryptophan

The experiments were performed in 0.066 M phosphate buffer at pH 7.50 ± 0.05 . K^0 = per cent hydrolysis per minute; C^0 = K^0 per mg. of protein N per cc. of test solution.

Enzyme concentration	Time	Hydrolysis	K^0	C^0	C^0 , average
<i>protein N per cc.</i>	<i>min.</i>	<i>per cent</i>			
0.158	180	27	0.15	950	1010
	240	39	0.16	1010	
	330	54	0.16	1010	
	360	60	0.17	1080	
0.316	60	19	0.32	1010	1040
	90	31	0.34	1080	
	120	39	0.33	1040	
	150	51	0.34	1080	
	180	59	0.33	1040	
	210	67	0.32	1010	
0.474	60	27	0.45	950	920
	120	51	0.43	910	
	180	75	0.42	890	
0.632	45	27	0.60	950	935
	60	35	0.58	920	
	90	54	0.60	950	
	120	70	0.58	920	
0.948	30	24	0.80	840	920
	45	42	0.93	980	
	60	53	0.88	930	
	90	80	0.89	940	
1.264	15	18	1.20	950	970
	30	39	1.30	1030	
	45	51	1.13	890	
	60	70	1.17	930	

The relative rates of hydrolysis for various substrates may be compared by their specific proteolytic coefficients, C , which are defined as the velocity constants for an enzyme concentration of 1 mg. of protein N per cc. of test solution. The coefficients in Table II indicate that CTA is hydrolyzed 15.7 times more rapidly than CTG. It is of interest that the ratio for carbobenzoxyglycyl-L-alanine (CGA) and carbobenzoxyglycylglycine (CGG) as

computed from the C values (first order) given by Stahmann, Fruton, and Bergmann (6) is $0.038/0.0024$ or 15.8. It may be recalled that Bergmann and Fruton (5) postulated that the relative rates of hydrolysis of two substrates with different R'' groups should be the same regardless of the nature

TABLE II

Action of Carboxypeptidase on Various Substrates

The solutions were buffered by 0.066 M phosphate at pH 7.5 ± 0.1 . C^0 is the zero order velocity constant per mg. of protein-N per cc., while C_1 is the corresponding first order constant.

Substrate	Protein N per cc. test solution	Time	Hydro- lysis	C^0	C_1
	mg.	hrs.	per cent		
Carbobenzoxy-L-tryptophylglycine	0.316	0.5	13	1.4	0.0068
		1.0	27	1.4	0.0073
		1.5	46	1.6	
		2.0	50	1.3	
		2.5	61	1.3	
Carbobenzoxy-L-tryptophyl-L-alanine	0.0316	0.5	23	24.0	0.120
		1.0	41	21.0	0.120
		1.5	60	21.0	
		2.0	68		
Carbobenzoxy-L-tryptophyl-L-tyrosine	0.00054	0.5	12		
		1.0	*		
Carbobenzoxy-L-tryptophyl-L-tryptophan	0.00316	0.5	39	-	
		1.0	51		
		1.5	80		
		20.0	140†		
Carbobenzoxy-L-tryptophyl-L-proline	0.268	1.0	8		0.0023
		2.0	15		0.0022
		3.5	18		0.0015
		20.0	87		0.0028
Carbobenzoxyglycyl-L-tryptophanamide	0.0632	20.0	5		
Carbobenzoxyglycyl-L-thiazolidine-4-carboxylic acid	0.316	20.0	20		
Carbobenzoxyglycylhydroxy-L-proline	0.316	20.0	2		
Carbobenzoxyglycyl-L-phenylalanine	0.000107				14

* Tyrosine crystallization.

† 100 per cent corresponds to the complete hydrolysis of one peptide bond.

of R' . Apparently, this postulate holds here, although the kinetics differ for the corresponding carbobenzoxytryptophyl and carbobenzoxyglycyl compounds.

Although the hydrolysis of the tryptophan compounds is best described

as following the kinetics of a zero order reaction, the initial rates can be computed equally well for a first order reaction. This has been done in order to have some comparison with the rates for other compounds, although it is evident that this procedure affords only an approximation. C (first order) for CTA is 0.120 and C (first order) for CTG is 0.0068. The ratios of the first order proteolytic coefficients are then $C_{CTA}/C_{CGA} = 3.2$, and $C_{CTG}/C_{CGG} = 2.8$. The good agreement between these quotients allows some confidence in the statement that the substitution of a tryptophyl for a glycyl residue in the position of R' increases the sensitivity of the substrate about 3 times. This is of particular interest since previous work (5) has shown that alanyl or glutamyl residues in this position decrease the rates as compared to glycyl residues.

Assuming then that the Bergmann-Fruton postulates hold, it is possible to calculate the relative sensitivity of CGT as compared to carbobenzoxyglycyl-L-phenylalanine (CGP), the most sensitive known substrate for this enzyme. Since $C_{CGP}/C_{CGA} = 13/0.038$ (from Stahmann *et al.* (6)), the zero order constants $C_{CTA}/C_{CTG} = 22.0/980$ and $C_{CGA}/C_{CTA} = 1/3.2$, the equations are readily combined to give

$$\frac{C_{CGP}}{C_{CGT}} = \frac{C_{CGP}}{C_{CGA}} \cdot \frac{C_{CGA}}{C_{CTA}} \cdot \frac{C_{CTA}}{C_{CGT}} = \frac{13 \times 1 \times 22.0}{0.038 \times 3.2 \times 980} = 2.4$$

A direct comparison has also been made by taking the hydrolysis for the first reading at each of the six different enzyme concentrations given in Table I for CGT and computing C_{CGT} (first order). The results were 4.9, 4.7, 4.9, 4.9, 4.2 and 4.6, giving an average of 4.7. $C_{CGP}/C_{CGT} = 13/4.7$ or 2.8 as compared with the value 2.4 calculated indirectly. The results suggest that the Bergmann-Fruton concept is valid regardless of the nature of the reaction kinetics obtained in particular experiments. It is also evident that the high affinity of carboxypeptidase for substances containing an aromatic ring is observed not only for the phenylalanine and tyrosine compounds but for tryptophan as well.

It is clear from our data that carboxypeptidase is particularly sensitive to the nature of the amino acid in the R'' position and much less sensitive to R' . Change from a tryptophyl to a glycyl residue in R' causes only a 3-fold alteration in the rate of action on the sensitive peptide bond as judged by the ratio of the proteolytic coefficients of CTG to CGG. However, the isomeric compound (CGT) containing the same amino acids shows a difference in sensitivity to the enzyme when compared to CGG of 4.7/0.0024 or 1960 times. Obviously, the nature of the residue possessing the free carboxyl group is the main determinant of sensitivity of the substrate to the enzyme.

Carbobenzoxyglycyl-L-tryptophanamide is not hydrolyzed at a signifi-

cant rate by carboxypeptidase. This is in agreement with the results of other investigators who have noted that a free terminal carboxyl group is necessary for the action of this enzyme (4).

Compounds which do not possess a free hydrogen at the sensitive peptide bond have been reported to be resistant to carboxypeptidase action. This was found by Stahmann, Fruton, and Bergmann (6) using the substrates carbobenzoxyglycylsarcosine and carbobenzoxyglycyl-L-proline. It has now been observed that carbobenzoxy-L-tryptophyl-L-proline (CTP) is slowly hydrolyzed (Table II) by carboxypeptidase. This has been detected only because the introduction of the tryptophyl residue increases the sensitivity of the substrate 3-fold as compared to the corresponding glycine compound. Thus, CTP is hydrolyzed at about the same rate as CGG. It would be expected that the specific proteolytic coefficient of carbobenzoxyglycyl-L-proline would be about 0.0007, which would not be measurable at the usual enzyme concentrations. Similar observations were made with carbobenzoxyglycylhydroxy-L-proline (7) and carbobenzoxyglycyl-L-thiazolidine-4-carboxylic acid; these showed only slight hydrolysis in 20 hours.

The titration data on carbobenzoxy-L-tryptophyl-L-tryptophan indicate a greater hydrolysis than that expected for a single peptide bond (Table II). Tests with carbobenzoxy-L-tryptophan and carbobenzoxy-DL-tryptophan indicate that these are split by carboxypeptidase (Table III).

Studies of mixtures of carbobenzoxyglycyl-L-thiazolidine-4-carboxylic acid with CTG (Table III) or with CGP did not show any inhibition of the enzyme, indicating that the affinity of carboxypeptidase for this compound with a ring nitrogen is very slight indeed.

The author gratefully acknowledges the technical assistance of Mrs. Toshiko Shimizu. He is also indebted to Dr. H. T. Hanson for his help with the preparation of carboxypeptidase and to Dr. M. D. Armstrong for the sample of L-thiazolidine-4-carboxylic acid.

EXPERIMENTAL

The crystalline carboxypeptidase was prepared by the method of Anson¹ (8) and recrystallized five times as described by Neurath, Elkins, and Kaufman (9). The enzymatic experiments were carried out in 2.5 cc. volumetric flasks at a temperature of 25°. Hydrolysis was measured on 0.2 cc. samples by the titration method of Grassmann and Heyde (10). The substrate was present in a concentration of 0.05 M with the exception of the racemic substrates which were used at 0.1 M.

It was found that several tryptophan derivatives did not always give reliable analyses for nitrogen by the Kjeldahl method. Hence, all of the analyses reported below were performed by the micro-Dumas method.

¹ Dr. M. L. Anson was kind enough to furnish seed crystals of the enzyme.

Likewise, carbobenzoxyglycyl-L-thiazolidine-4-carboxylic acid gave low nitrogen values by the Kjeldahl method. Most of the microanalyses were performed by Dr. A. Elek of Los Angeles. The melting points were obtained in open capillary tubes; they are uncorrected values.

Carbobenzoxyglycyl-L-tryptophan—To a solution of 7 gm. of L-tryptophan in 36 cc. of M NaOH at 0°, there were added with cooling and shaking an additional 36 cc. of M NaOH and 8 gm. of carbobenzoxyglycyl chloride. On acidification with 5 M HCl, the compound crystallized as a solid mass of

TABLE III
Action of Carboxypeptidase

The conditions of these experiments were the same as those described in Tables I and II.

Substrate	Protein N per cc. test solution	Time	Hydrolysis	K°	C°
	mg.	hrs.	per cent		
Carbobenzoxy-L-tryptophan (0.05 M)	0.268	0.5	16	0.53	
		1.0	31	0.52	
		1.5	58	0.64	
		2.0	73	0.61	
		2.5	78	0.52	2.1
Carbobenzoxy-DL-tryptophan (0.1 M)	0.268	1.5	44	0.44	
		3.0	64	0.36	1.5
Carbobenzoxyglycyl-L-tryptophan	0.000632				935
Carbobenzoxyglycyl-L-tryptophan (0.05 M) + carbobenzoxyglycyl-L- thiazolidine-4-carboxylic acid (0.05 M)	0.000632	1.0	36		950
		1.5	54		950
		2.0	69		920

fine needles. Yield, 12.7 gm. On recrystallization from ethyl acetate-petroleum ether, rosettes of fine needles were obtained; m.p. 142°.

$C_{21}H_{21}O_5N_3$. Calculated. C 63.8, H 5.4, N 10.6
395.4 Found. " 63.7, " 5.4, " 10.6

Carbobenzoxyglycyl-L-tryptophanamide—To a dry ether solution of tryptophan methyl ester from 4.4 gm. of the hydrochloride, there were added in two portions 4 gm. of carbobenzoxyglycyl chloride at 0°. Following the addition of the second portion, the solution was shaken for 20 minutes with 50 cc. of a saturated solution of $KHCO_3$. The ether solution was washed with water, dilute HCl, again with water, dried over Na_2SO_4 , and concentrated *in vacuo* repeatedly with methanol. The syrup was then allowed to stand for 2 days at room temperature in a solution of methanol previously saturated with NH_3 gas at 0°. The amide crystallized partially after re-

peated concentration with methanol. It was recrystallized from ethyl acetate-petroleum ether. Yield, 2.7 gm. The compound softens at 117–121°; m.p. 145°.

$C_{21}H_{23}O_4N_4$	Calculated.	C 64.0, H 5.6, N 14.2
394.4	Found.	" 63.9, " 5.7, " 14.3

Carbobenzoxy-L-tryptophylglycine

Carbobenzoxy-L-tryptophan—To a solution of 5.1 gm. of L-tryptophan in 25 cc. of *M* NaOH at 0°, there were added with cooling and shaking 4.2 gm. of carbobenzoxy chloride and 25 cc. of *M* NaOH. 1 hour after the last addition, the solution was acidified to Congo red with 5 *M* HCl. The compound crystallized in rosettes of fine elongated plates. Yield, 8.3 gm. In various experiments, the yield ranged from 85 to 98 per cent of theory. After recrystallization from ethyl acetate-petroleum ether, the melting point was 126°.

$C_{19}H_{19}O_4N_3$	Calculated.	C 67.4, H 5.4, N 8.3
338.4	Found.	" 67.7, " 5.4, " 8.5

Carbobenzoxy-L-tryptophyl Chloride—To a suspension of 7.6 gm. of carbobenzoxy-L-tryptophan in 100 cc. of anhydrous ether at 0°, there were added 5.4 gm. of PCl_5 . After cooling and shaking for about 30 minutes, the acid chloride crystallized spontaneously in a solid mass of elongated fine needles. About 200 cc. of cold anhydrous petroleum ether were added, and the mixture stirred for a few minutes. The compound was filtered, washed with petroleum ether, and air-dried for 20 minutes on a porous plate. The yield was quantitative. M.p. 75° with decomposition. The compound is extremely insoluble in ether, petroleum ether, and water. It is readily soluble in ethyl acetate which was used as the solvent in various coupling reactions.

Carbobenzoxy-L-tryptophylglycine—The above acid chloride was added in 10 cc. of ethyl acetate to a solution of 2 gm. of glycine in 25 cc. of *M* NaOH at 0° with an additional 20 cc. of *M* NaOH. The aqueous solution was washed with ethyl acetate and acidified to Congo red with concentrated HCl. Yield, 6.7 gm. Triangular plates were obtained after recrystallization from ethyl acetate-petroleum ether; m.p. 156°.

$C_{21}H_{21}O_4N_3$	Calculated.	C 63.8, H 5.4, N 10.6
395.4	Found.	" 63.7, " 5.4, " 10.7

Carbobenzoxy-L-tryptophyl-L-alanine—This was prepared as described for the above glycine compound with the acid chloride from 3.4 gm. of carbobenzoxy-L-tryptophan and 1 gm. of L-alanine. Yield, 4.0 gm. The compound was obtained as long needles with the correct composition after

recrystallization from ethyl acetate-petroleum ether. M.p. 93–99°. After recrystallization from hot water, the melting point rose to 165°.

$C_{22}H_{23}O_5N_3$.	Calculated.	C 64.6, H 5.7, N 10.3
409.4	Found.	" 64.4, " 5.7, " 10.1

Carbobenzoxy-L-tryptophyl-L-proline—This was prepared as for the above compounds with the acid chloride from 3.4 gm. of carbobenzoxy-L-tryptophan and 1.2 gm. of L-proline. Yield, 4.4 gm. The substance was recrystallized by slow addition of petroleum ether to an ethyl acetate solution of the compound. The product gave the correct analysis but had a poor melting point. After recrystallization from methanol-water, the compound melted at 128–130°.

$C_{24}H_{25}O_5N_3$.	Calculated.	C 66.2, H 5.8, N 9.7
435.5	Found.	" 66.0, " 6.0, " 9.6

Carbobenzoxy-L-tryptophyl-L-tryptophan—The coupling was performed as above with the acid chloride from 3.4 gm. of carbobenzoxy-L-tryptophan and 2.0 gm. of L-tryptophan. The sodium salt immediately crystallized as fine needles from the alkaline solution. On acidification to Congo red, 5.2 gm. of needles were obtained. The compound was dissolved in a mixture of warm ethyl acetate and methanol; it crystallized on addition of petroleum ether. M.p. 207° with slight decomposition.

$C_{30}H_{33}O_5N_4$.	Calculated.	C 68.7, H 5.9, N 10.7
524.6	Found.	" 68.6, " 5.5, " 10.6

Carbobenzoxy-L-tryptophyl-L-tyrosine—This was prepared in the same manner with 1.8 gm. of L-tyrosine. The aqueous suspension of the insoluble sodium salt was acidified, and the compound was taken up in ethyl acetate. The solvent was evaporated *in vacuo*, and the substance solidified on standing with water. Yield, 1.7 gm. The product softened in the neighborhood of 90–100°, and melted poorly at about 135–140° with decomposition.

$C_{28}H_{27}O_5N_3$.	Calculated.	C 67.1, H 5.4, N 8.4
501.5	Found.	" 67.2, " 5.6, " 8.1

Carbobenzoxy-DL-tryptophan—This was prepared in the same manner as the L compound described above. The melting point was 169–170° for the racemic compound as compared to 126° for the L-derivative.

$C_{19}H_{19}O_4N_2$.	Calculated.	C 67.4, H 5.4, N 8.3
338.4	Found.	" 67.5, " 5.5, " 8.3

Carbobenzoxyglycyl-L-thiazolidine-4-carboxylic acid—To a solution of 6.7 gm. of thiazolidine-4-carboxylic acid (11) in 50 cc. of M NaOH at 0°, there were added in portions with cooling and shaking an additional 60 cc. of M

NaOH and an ethereal solution of carbobenzoxyglycyl chloride prepared from 12.6 gm. of carbobenzoxyglycine. Half an hour after the final additions, the solution was washed with ether and acidified to Congo red with concentrated HCl. The substance was extracted into ether, dried, and concentrated *in vacuo*. Crystals were obtained on standing with a small volume of ether in the cold. Yield, 7.5 gm. The compound was obtained as plates on recrystallization from hot ethyl acetate; m.p. 109–110°. An additional crop of crystals of lower melting point (103–106°) was obtained by addition of petroleum ether to the mother liquor.

$C_{14}H_{16}O_4N_2S$	Calculated.	C 51.8, H 5.0, N 8.6, S 9.9
324.4	Found.	" 51.6, " 5.2, " 8.5, " 10.2

SUMMARY

1. Crystalline pancreatic carboxypeptidase hydrolyzes carbobenzoxy-L-tryptophylamino acids about 3 times more rapidly than the corresponding glycyl compounds. Carbenzoxyglycyl-L-phenylalanine is hydrolyzed by this enzyme about 2.6 times more rapidly than carbobenzoxyglycyl-L-tryptophan. The hydrolysis of various tryptophan substrates under standard conditions proceeds with the kinetics of a zero order reaction, in contrast to the first order reaction usually found with similar derivatives of other amino acids.

2. The synthesis of carbobenzoxy derivatives of tryptophan and tryptophan peptides and of carbobenzoxyglycyl-L-thiazolidine-4-carboxylic acid is described.

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A COMPARISON OF THE INFLUENCE OF 2,4-DINITROPHENOL ON THE OXYGEN CONSUMPTION OF RAT BRAIN SLICES AND HOMOGENATES*

By CLARENCE NORMAN PEISS AND JOHN FIELD

*(From the Department of Physiology, Stanford University School of Medicine,
Stanford University, California)*

(Received for publication, March 17, 1948)

It is well established that the oxygen consumption of various types of intact cells is augmented when low concentrations of 2,4-dinitrophenol (DNP) or certain other substituted phenols are added to the medium in which the cells are suspended. Slightly higher concentrations depress oxygen consumption. This has been shown for yeast (1-6), luminous bacteria (7), various marine eggs (8-14), orthopteran embryos (15), mammalian sperm (16-19), and several amphibian (20) and mammalian (21-25) tissues. Similar observations have been reported in some studies on glycolysis (*cf.* (26)). The augmentation phase of the concentration-action curves (*cf.* (27)) which depict the effect of graded concentrations of DNP (and certain other substituted phenols) on oxygen consumption appears to depend upon factors associated with cell structure or rendered inactive by dilution (*cf.* (28)), because it has not been observed in cytolyzed material (29) or in cell-free preparations of various enzymes directly concerned in cell respiration (13, 30-32). Recently Reiner (33) has described a rat brain homogenate, reinforced with the known co-factors of glycolysis and with nicotinamide, cytochrome *c*, and fumarate, which is capable of oxidizing glucose. Moreover Reiner has defined the conditions for maximum activity in this system. In order to obtain further information regarding the action of the substituted phenols in augmenting cell respiration, which is still imperfectly understood (26, 34, 35), we have compared the influence of graded concentrations of DNP on the oxygen consumption of rat brain slices and homogenates with glucose as the fuel in each case. The results are presented in this paper.

Methods

Tissue was obtained from twelve adult albino rats of the Slonaker-Wistar strain. After decapitation the brain was rapidly excised and placed in a small chilled beaker standing in a tray of cracked ice. Beaker and tray were transferred to a moist cold box (36). The brain was then removed

* Supported in part by a grant from the Medical Research Fund of Stanford University School of Medicine.

from the beaker and placed on a paraffined stopper. Adherent blood was removed with filter paper and meningeal tissue was stripped off with bone forceps. Cerebral cortex slices were prepared by the Lucite templet method (37) by means of a clean, dry safety razor blade (38, 39). Tissue sections were removed from the cutting blade with fine forceps, and placed in a Petri dish which stood on the surface of a tray of cracked ice. This dish was kept humid by means of a piece of filter paper moistened with Ringer's solution which was stuck on the under surface of the lid. The lid was so placed that the dish was partly open to receive tissue. The slices were arranged in a number of small piles corresponding to the number of flasks to be loaded. When a suitable quantity of tissue was on hand, the dish was closed and the ice tray and dish were removed from the cold box. The slices were rapidly weighed on a micro torsion balance. The usual load placed in a 15 ml. respirometer flask was about 50 mg. of wet weight. Control experiments showed that with sample weights ranging from 20 to 90 mg. respiration varied directly with tissue weight. Aliquot samples (duplicate or triplicate) were placed in small weighing bottles and dried to constant weight in an electric oven at 105°. After loading with tissue, the respirometer flasks, which already contained the necessary solutions (chilled), were attached to the corresponding manometers and oxygenated at room temperature. They were then placed in a water bath maintained at $37.5^{\circ} \pm 0.01^{\circ}$. By this procedure the brain tissue is kept in a cold moist environment from the time of excision until adequate oxygen is available. Thus imbalance between the anaerobic and aerobic aspects of metabolism is minimized (40). The suspension medium was Krebs' Ringer-phosphate, pH 7.35, containing 0.011 M glucose. The gas phase was oxygen. Results are expressed in the conventional Q notation. Thus Q_{O_2} denotes microliters of oxygen consumed, measured under standard conditions, per mg. of dry weight of tissue per hour.

The homogenates were prepared by means of an all glass Potter-Elvehjem (28) homogenizer in ice-cold double distilled water. The system for the homogenate work was essentially that of Reiner (33). The following constituents were used in the final (vessel) concentrations given: glucose 0.028 M, hexose diphosphate (HDP) 0.005 M, adenosine triphosphate (ATP) 0.0007 M, coenzyme I (DPN) 0.001 M, potassium fumarate 0.0016 M, nicotinamide 0.04 M, magnesium chloride 0.008 M, cytochrome *c* 0.00006 M, KH_2PO_4 - K_2HPO_4 buffer, pH 7.4, 0.04 M. The HDP, ATP, and DPN were commercial preparations from the Schwarz Laboratories. The former two were supplied as the barium salts and were converted to the potassium salts shortly before use. Potassium fumarate was prepared by neutralizing fumaric acid with potassium hydroxide. Cytochrome *c* was a commercial preparation (Tremond). All solutions were freshly prepared before each

experiment. Suitable concentrations of 2,4-dinitrophenol (DNP) were made up in Krebs' Ringer-phosphate for the tissue slice experiments and in 0.04 M phosphate buffer, pH 7.4, for the homogenate runs. When used, DNP was added from the side arms of the respirometer flasks 30 minutes after the end of the thermoequilibration period, thus allowing each preparation to serve as its own control for the initial 30 minutes. In all cases steady states of oxygen consumption were observed during the control period. On addition of DNP the maximum effect was very rapidly attained and remained at the same level of intensity for a considerable time. The degree of augmentation or inhibition was calculated from steady state data before and after addition of DNP.

Results

The effect of graded concentrations of DNP on the oxygen consumption of rat cerebral cortex slices in Krebs' Ringer-phosphate solution and of reinforced rat brain homogenate in phosphate buffer at 37.5° is shown in Fig. 1. An arbitrary value of 1.00 has been assigned to the rate of respiration during the control period (before addition of DNP). It is evident from inspection of Fig. 1 that under the conditions of these experiments the respiration of cerebral cortex slices was augmented in the presence of concentrations of DNP ranging from 4.46×10^{-6} to 8.92×10^{-6} M and was depressed by concentrations of 1.12×10^{-4} M or higher. In the rat brain homogenate the picture was quite different. The concentrations of DNP which augmented respiration in the slices, *i.e.*, 4.46×10^{-6} to 8.92×10^{-6} M, inhibited oxygen consumption in the homogenate, the degree of inhibition increasing as the concentration of DNP rose. It seemed possible that an augmentation phase in homogenate respiration might occur at lower levels of DNP than in the slice. To see whether this was so we examined the effect of graded concentrations of DNP ranging down to 1.11×10^{-6} M. No augmentation phase was found. Concentrations of DNP below 4.46×10^{-6} M did not influence homogenate respiration. Thus when oxygen consumption is measured, with glucose as fuelstuff, the concentration-action curve of DNP is diphasic for cerebral cortex slices, which are essentially preparations of intact cells, and monophasic for brain homogenates, in which practically no intact cells were found on microscopic examination (although many apparently undamaged nuclei were present).

There is one interesting feature of the data which is not brought out in Fig. 1 where the values of respiration are given on a relative basis. This feature is the striking similarity between the maximum rate of respiration in cerebral cortex slices in the presence of DNP (in "optimum" concentrations for respiration) and the respiratory rate in homogenates without DNP. Thus the rate of oxygen consumption of cerebral cortex slices in

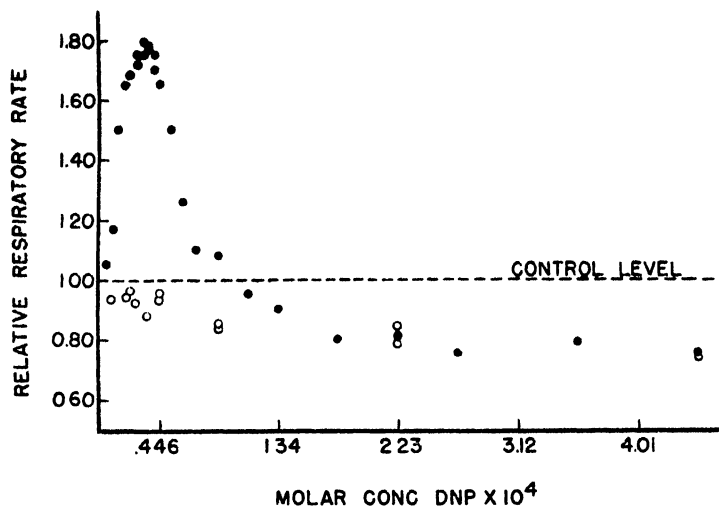


FIG. 1. Concentration-action curve showing relative oxygen consumption of cerebral cortex slices (●) and reinforced brain homogenates (○) as a function of the concentration of 2,4-dinitrophenol. An arbitrary value of 1.00 has been assigned to the control rate (no dinitrophenol present). For details see the text.

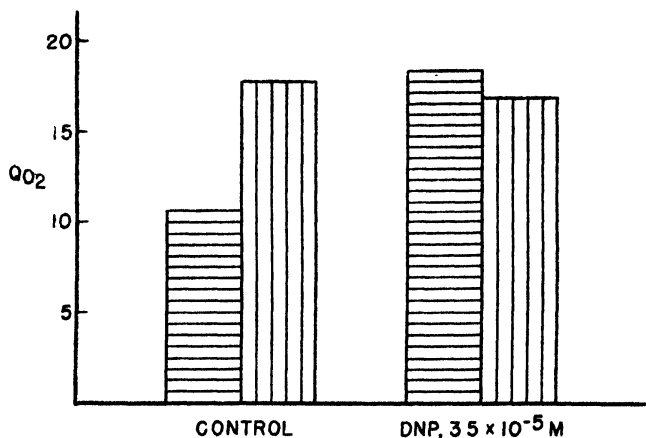


FIG. 2. Block diagram to illustrate the effect of the concentration of 2,4-dinitrophenol usually evoking maximum increase in the respiration of cerebral cortex slices on the respiration of such slices and of reinforced brain homogenates. Blocks with horizontal and vertical bars represent Q_{O_2} values of cerebral cortex slices and brain homogenates respectively. No dinitrophenol was present in the controls.

the presence of optimum concentrations of DNP corresponded to Q_{O_2} values of 17.5 to 19.9, while in homogenates the Q_{O_2} during the control period (before addition of DNP) ranged from 16.4 to 20.4 with a mean of

17.7 (twenty-six runs). In other words the two systems appeared to have approximately the same respiratory ceiling. This is shown in Fig. 2.

It must be pointed out that the comparison drawn in Fig. 2 is not a simple one. It involves two quite different systems. On the one hand we are dealing with cerebral cortex slices, consisting mostly of intact cells, suspended in an "extracellular phase" medium (*cf.* (38, 41)), in the presence of optimum concentrations of DNP; on the other hand with a system made up of disintegrated cells, with considerable dilution of cellular material (28), present in an approximation of an "intracellular phase" medium (38, 41) and reinforced with various factors important in glycolysis and respiration. However, in the nature of the case no single medium could be used for this comparison (*cf.* (38)). It should be noted that the homogenate used was the "complete system" of Reiner (33) in which it appears that the conditions are such as to reveal the full potential respiratory capacity of brain tissue (33). It seems to us that the similarity in respiratory rate between this system and cerebral cortex slices in the presence of optimum concentrations of DNP is more likely to be meaningful than fortuitous.

DISCUSSION

Three major groups of observations bearing on the mechanism of augmentation of cell respiration by DNP are reported in this paper. These are (1) that optimum concentrations of DNP raise the rate of respiration in cerebral cortex slices to about the same level as in brain homogenates without DNP but reinforced to exhibit maximum respiratory activity (with glucose as the fuelstuff in each case); (2) that no augmentation of respiration is obtainable, under the conditions of these experiments, when DNP is added to homogenates; (3) that concentrations of DNP which augment the respiration of cerebral cortex slices produce a slight depression of respiration in homogenates.

On the basis of these observations it is suggested as a tentative working hypothesis that augmentation of cell respiration by DNP is due to inhibition of an enzyme "brake" or regulator (*cf.* (42) p. 178) by this agent; *i.e.*, that it is a matter of "deinhibition" rather than "stimulation." In so far as respiration itself is concerned the action of DNP appears to be uniformly inhibitory as shown by its effect on this process in the homogenate. Failure to observe augmentation of respiration in the homogenate treated with DNP may well be due to loss of the regulator by the dilution effect (*cf.* (28)) during preparation. The high respiratory rate of the untreated homogenate is in consonance with this suggestion.

The view that DNP and substituted phenols of like effect may increase cell respiration by inhibition of a regulator is not new (*cf.* (25, 30)). How-

ever, this view has been both strengthened and somewhat modified by the evidence presented here. Thus the hypothesis is supported by the observation that the ceiling for respiration is about the same, with glucose as a common fuelstuff, for intact brain cells in the presence of an optimum concentration of DNP and for brain homogenates containing no DNP, prepared so as to exhibit apparently maximum activity. It is modified by the observation that concentrations of DNP which augment respiration in the intact brain cell slightly inhibit respiration in the brain homogenate so that the augmentation phase represents the algebraic sum of the effect of inhibition of the regulator and of the respiratory process rather than simply inhibition of the former. In a tissue in which the latter effect predominates there would be no augmentation phase.

The view that augmentation of tissue respiration by DNP is basically an inhibitory phenomenon introduces unity into the concept of the biological activity of this substance and the substituted phenols having qualitatively similar effects. In general these agents inhibit enzyme activity, and the augmentation of the exergonic (43) processes, respiration and fermentation (*cf.* (26)), by low concentrations of DNP and the like was a unique and anomalous aspect of their action. Concentrations of the substituted phenols which augment or do not depress cell respiration appear to decrease the energy available for assimilation or work (*cf.* (34)). Thus such concentrations inhibit division in fertilized *Arbacia* eggs (8-13), multiplication of yeast (44), and growth and development in the frog (45), in a teleost (46), and in *Drosophila* (47). They also inhibit assimilation of carbohydrate (48) and of protein (49) in various types of cells, phosphate turnover (49), motility in sperm (16-18), ciliary movement in *Arenicola* larvae (50), and voluntary activity in the rat (51).

While a fair case can be made for the view that the effect of DNP and substituted phenols of like action is always inhibitory on the enzyme level, the evidence is only presumptive, not conclusive. The hypothesis would be much strengthened if the regulator could be identified, as in the case of the hexokinase reaction studied by Cori and Cori and their associates (52, 53). It might then be possible to depress the respiration of homogenates by addition of the regulator and to relieve this inhibition by addition of DNP. It is planned to deal with these questions in a subsequent investigation.

SUMMARY

1. The oxygen consumption of rat cerebral cortex slices in Krebs' Ringer-glucose solution is augmented when sufficient 2,4-dinitrophenol is added to the medium to give concentrations ranging from 4.46×10^{-4} to 8.92×10^{-5} M. Higher concentrations of DNP depress respiration.

2. Concentrations of DNP which augment respiration in cerebral cortex slices cause a slight inhibition of oxygen consumption in reinforced brain homogenates. Higher concentrations of DNP cause more marked inhibition. Concentrations of DNP lower than those augmenting respiration in cerebral cortex slices have no effect on homogenate respiration.

3. Concentrations of DNP evoking maximum increase in the respiration of cerebral cortex slices bring the oxygen consumption of the slices to about the same level as in reinforced homogenates untreated with DNP.

4. It is suggested that augmentation of cell respiration by DNP is a matter of "deinhibition" rather than "stimulation," and that the essential feature of the augmentation is inhibition of an enzyme "brake" or regulator.

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THE VERATRINE ALKALOIDS

XXVIII. THE STRUCTURE OF JERVINE

By WALTER A. JACOBS AND YOSHIO SATO

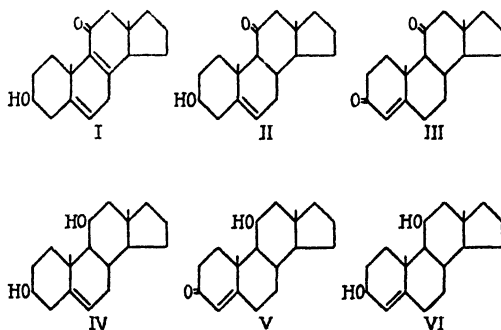
(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, April 14, 1948)

Since the previous communication (1) on jervine, new observations have been made which, with reinterpretation of some of the earlier data, now permit of certain conclusions in regard to its structure. Most satisfactory, as presented in Formula I, is that of a 3(β)-hydroxy-11-keto- $\Delta^{5,8(9)}$ -steroidal base in which the secondary basic group is contained on the iso-octyl side chain. The probable oxidic linkage appears also to be attached at least in part to the side chain but its exact location remains to be determined.

In the course of the earlier work to identify the double bond still present in dihydrojervine the attempt was made to convert this derivative, as with jervine itself, with aluminum tert-butoxide to the Δ^4 -ketone and the latter in turn with aluminum isopropoxide to a possible Δ^4 -dihydrojervine. The last stage yielded a number of fractions of a crystalline hydrochloride which gave no Rosenheim test or only a weak one with trichloroacetic acid. At the time this appeared to support the possibility of hydrogenation of the Δ^5 double bond of jervine in the formation of dihydrojervine. However, other evidence has since been accumulated which makes this untenable. Much more clear cut was the experience with β -dihydrojervinol (Formula IV) in which the carbonyl group of dihydrojervine has been reduced with sodium and butanol (1). The dihydrojervine (Formula II) used for reduction to β -dihydrojervinol was first treated with HCl to remove contaminating jervine by isomerization. β -Dihydrojervinol was smoothly converted by the Oppenauer method to the crystalline Δ^4 -3-keto derivative $C_{27}H_{41}O_2N$ (Formula V) which readily yielded an oxime. The ultraviolet absorption spectrum obtained with the Δ^4 -ketone is shown in Fig. 1, together with that of the original β -dihydrojervinol. The ketone was in turn readily reduced with aluminum isopropoxide to the crystalline Δ^4 -3-hydroxy derivative $C_{27}H_{43}O_3N$ which, contrary to β -dihydrojervinol, now gave a pronounced Rosenheim reaction (Formula VI). No attempt has been made as yet to determine the configuration of the 3-hydroxyl group of the substance isolated or to separate possible epimers, if a mixture. However, the product was isolated in several fractions which closely agreed in properties.

This series of reactions parallels the experience with jervine itself and with other 3-hydroxy- Δ^5 -steroids. It has been assumed that the original carbonyl group of jervine as such, or after its reduction with sodium to hydroxyl, has not participated in these reactions. This was especially indicated by the general unreactivity of the carbonyl group of jervine. Aside from its reduction with sodium, this inertness was shown by the failure of all more recent attempts to cause it to react with ketone reagents. Similarly jervine was recovered unchanged after the usual treatment with aluminum isopropoxide. This behavior strongly indicates a CO group at position 11 (2, 3). As a possible additional check for the 11-keto group, the behavior was studied of the ease of acylation of the OH group formed from it by reduction. However, β -dihydrojervinol in pyridine at room temperature with acetic anhydride yielded quite readily *N*-acetyldihydrojervinol diacetate. As in the case of sarmentogenin this ease of acylation



indicates a less hindered configuration for the new hydroxyl group (3) than that found with the adrenal 11-hydroxy steroids (2).

In the earlier paper absorption spectrum studies with dihydrojervine were discussed. Due to the abnormal results obtained, the possibility of contamination with unchanged jervine was considered but discarded because of the failure of repeated attempts to change these results by certain purification procedures. However, this has since been found to be incorrect by the use of a relatively large amount of catalyst for the first step in the hydrogenation of jervine. A dihydrojervine was obtained with properties essentially as previously recorded with the exception, however, that the ultraviolet absorption data obtained with it (Fig. 1) now no longer showed the strong absorption in the shorter wave-lengths. The curve closely resembles that previously obtained from dihydrojervine which had been recovered after treatment with HCl to remove possible contamination with jervine. However, the presence of some absorption in the shorter wave-lengths suggests the persistence of slight contamination. The peak which persists at about $300\text{ m}\mu$ is due to carbonyl group absorption.

In view of the normal behavior of β -dihydrojervinol, we returned to a study of dihydrojervine in which the same acid-treated material was used. With aluminum tert-butoxide and acetone a product resulted which was purified through alumina and gave in good yield a rather low melting (131 – 132°) crystalline substance. The ultraviolet absorption spectrum of the latter (Fig. 1), contrary to that of dihydrojervine, now yielded the absorption to be expected of Δ^4 -dihydrojervone as shown in the partial Formula III.

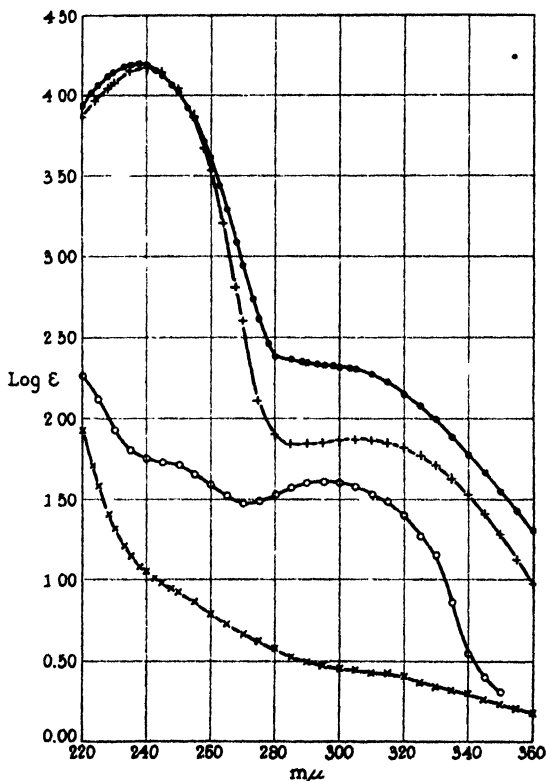


FIG. 1. ○ = dihydrojervine; ● = Δ^4 -dihydrojervone; + = Δ^4 - β -dihydrojervonol; × = β -dihydrojervinol; all in ethanol.

The latter was further characterized by its *oxime*. The next step with aluminum isopropoxide, however, again gave ambiguous results. The reaction product isolated as the base in a number of crystalline fractions failed in largest amount to give the expected Rosenheim test. Only a very small fraction was obtained which gave a sufficiently strong, although abnormal, color with trichloroacetic acid. In addition, analytical discrepancies (0.5 to 1 per cent high in C) persisted in attempts to characterize

the various fractions. Because of these difficulties a report of the experimental details of this part of the investigation will be deferred, pending the outcome of a further study planned to explain the anomalous results.

In the course of our work a personal communication was received from D. H. R. Barton who correctly questioned the earlier abnormal ultraviolet absorption curve attributed to dihydrojervine (and since rejected by us) as being due to a $\Delta^{\alpha,\beta}$ -ketonic type of absorption. He also called attention to previously published data of this laboratory (1) as follows: If one corrected for possible contamination of the dihydrojervine used with about 4 per cent of jervine, its molecular rotation would be -341° . Since that observed with tetrahydrojervine was -90° , the observed change in molecular rotation was $+251^\circ$ on passing from the former to the latter. Similarly from our data the hydrogenation of β -dihydrojervinol with its molecular rotation of -17° to tetrahydrojervinol of molecular rotation $+211^\circ$ shows a change of $+228^\circ$. Both of these results approximate the change in molecular rotation of $+240^\circ$ shown by Barton (4) to be characteristic for the hydrogenation of a Δ^5 -steroid bond.

We have also found that the expected $3(\beta)$ -hydroxyl as well as the Δ^5 -stenol character of jervine which does not form an insoluble digitonide has been confirmed by the application of the method of Barton (5) to *N*-acetyljervine ($[\alpha]_D^{27} = -118.5^\circ$ ($c = 1.07$ in chloroform)). A comparison of the molecular rotation of the latter with those of *N*-acetyljervine acetate, $[\alpha]_D^{27} = -114^\circ$ ($c = 1.08$ in chloroform), and *N*-acetyljervine benzoate, $[\alpha]_D^{27} = -81^\circ$ ($c = 0.96$ in chloroform), has shown that the molecular rotations are respectively -553° , -580° , and -463° , or a Δ_1 of -27° and a Δ_2 of $+91^\circ$. The Δ_1 and Δ_2 values of Barton are respectively -35° and $+81^\circ$.

The persistence of the Δ^5 bond in β -dihydrojervinol (and also in α -dihydrojervinol) shows that this bond in dihydrojervine and jervine resists reduction with sodium and thus conforms to the behavior of the usual 3 -hydroxy- Δ^5 -steroids first shown with cholesterol itself (6, 7).

In earlier work it was found that the highly hydroxylated tertiary bases cevine (8), germine (9), and protoverine (10) are reduced with sodium to dihydro bases. Since this differs from the usual behavior for a Δ^5 -stenol, a recent check was made on the behavior of rubijervine, isorubijervine, and solanidine. As was to be expected, they were not reduced by this method.¹ A further difference between the cevine group and the simpler bases as already recorded was the resistance of the former to the usual hydrogenation procedure (except in the case of cevine with nickel). However, after isomerization with alkali to iso bases (9, 10), presumably due

¹ The statement previously made ((1) p. 639, paragraph 2) conveys an incorrect and unintended meaning in this regard and should be discarded.

to a bond shift, such hydrogenation readily occurred. It is probable that these natural polyhydroxy bases are $\Delta^{8(10)}$ -stenols and the iso bases are Δ^{14} derivatives. A suggestion of this was seen by the use of the method of molecular rotation differences of Barton (11).

On passing from isogerminine of $[\alpha]_D^{25} = -46.5^\circ$ ($c = 1.01$ in 95 per cent ethanol) to the value recently found for dihydroisogerminine of $[\alpha]_D^{27} = -51.4^\circ$ ($c = 1.01$ in 95 per cent ethanol), a Δ of -26° is obtained which agrees with the Δ value of -24° given by Barton for a Δ^{14} -stenol (β -stenol). A similar result, *viz.* $\Delta = -27^\circ$, is obtained by using the rotations of these substances found in pyridine respectively of $[\alpha]_D^{28} = -56^\circ$ ($c = 1.00$) and $[\alpha]_D^{28} = -61^\circ$ ($c = 0.95$).

With isoprotoverine and dihydroisoprotoverine (10), because of sparing solubility, the previously reported rotations were taken only in pyridine. A more recent value for the former also in this solvent was $[\alpha]_D^{28} = -44^\circ$ ($c = 0.94$) and the rotation for dihydroisoprotoverine recalculated for the dried substance was $[\alpha]_D^{28} = -53^\circ$. From these figures the value obtained for Δ is -48° . Although not as close as in the case of isogerminine to the above Δ value, it also suggests a β -stenol type for isoprotoverine.

As a final point, the identity of the groups involved in the isomerization of jervine with acid to isojervine becomes of interest. Previously (1) the possibility of the formation of a new hydroxyl group by cleavage of the oxidic grouping was discussed. However, the stability of dihydrojervine to acid and the ready acetylation of the new hydroxyl group of isojervine to form an N-acetyljervine diacetate (1) suggest that isojervine may result from the enolization of the 11-CO group and conjugation of the new double bond to form a possible benzenoid Ring B. Although the latter is supported by the resistance of isojervine to hydrogenation, its previously published ultraviolet absorption curve does not conform to a simple benzenoid type. The study of this question will therefore be continued.

EXPERIMENTAL

Δ^4 - β -Dihydrojervinol—For the material used in this study 6 gm. of dihydrojervine obtained by the hydrogenation of jervine were treated with methanol (75 cc.) saturated with HCl at 0° and the paste of hydrochloride was allowed to come to room temperature. After an hour the salt was collected with a small amount of methanol and then decomposed with aqueous alkali for extraction with chloroform. After removal of solvent the dihydrojervine was crystallized from methanol. This material was reduced as previously described in butanol with sodium. The β -dihydrojervinol obtained after recrystallization melted sharply at 283.5 – 286° .

1.8 gm. were refluxed with 7.2 gm. of aluminum *tert*-butoxide dissolved

in a mixture of 170 cc. of benzene and 60 cc. of acetone for about 4 hours. After decomposition with dilute alkali the product was extracted with chloroform. The resin obtained by concentration of the washed and dried extract was dissolved in 50 cc. of acetone and allowed to stand for several days. 0.19 gm. of β -dihydrojervinol which crystallized was recovered. The mother liquor on concentration to 10 cc. yielded a crust of heavier crystals. After several hours this was collected with cold acetone. 0.9 gm. was obtained which sintered at 211° and melted at 219 – 223° . It separated without solvent as micro platelets on addition of ether to the benzene solution and after slight preliminary sintering melted at 221.5 – 223.5° .

$C_{27}H_{41}O_3N$. Calculated, C 75.82, H 9.67; found, C 75.56, H 9.42

The *oxime* was obtained in methanol solution with hydroxylamine hydrochloride and sodium acetate. The diluted mixture was made alkaline and extracted with chloroform. The oxime separated from 50 per cent methanol as flat micro needles which lost birefringence at 286 – 292° .

$C_{27}H_{43}O_3N_2$. Calculated, C 73.24, H 9.57; found, C 73.57, H 9.50

Δ^4 - β -Dihydrojervinol -- 0.3 gm. of Δ^4 - β -dihydrojervonol was dissolved in benzene and the solution was concentrated *in vacuo* to dryness to insure removal of all solvent. The residue was treated with a solution of aluminum isopropoxide in 25 cc. of isopropanol prepared from 1 gm. of aluminum. After refluxing for 2 hours, an additional 15 cc. of isopropanol were added and the mixture was very slowly distilled until the distillate no longer gave a Legal test. The mixture was further concentrated to small volume and, after cooling and decomposition with excess dilute NaOH, it was extracted with chloroform. The latter yielded on concentration a resin which was dissolved in methanol followed by careful dilution. When crystallization once began it was greatly aided by warming and formed a crust of small platelets. This fraction of 90 mg. on recrystallization from dilute methanol formed almost rhombic wedge-shaped aggregates of micro platelets which melted at 246 – 248° and were anhydrous. It gave a gradually developing pronounced deep purple color with trichloroacetic acid.

$[\alpha]_D^{25} = +54.5^\circ$ ($c = 1.03$ in chloroform)
 $C_{27}H_{43}O_3N$. Calculated. C 75.46, H 10.10
 Found. (a) " 75.16, " 10.09
 (b) " 75.81, " 10.14

The mother liquor of the above fraction contained most of the material and, although further dilution caused crystallization, it was found preferable to reextract with chloroform. The concentrated chloroform solution on addition of benzene caused a copious crystallization of aggregates of

pointed platelets which contained 1 mole of solvent. This fraction (0.16 gm.) melted at 243–246° and gave a Rosenheim test indistinguishable from the first fraction. For analysis it was dried at 110° and 0.2 mm.

$[\alpha]_D^{25} = +66^\circ$ ($c = 0.80$ in chloroform for dry substance)

$C_{27}H_{43}O_2N \cdot C_6H_6$. Calculated, C 15.38; found, 14.97

$C_{27}H_{43}O_2N$. Calculated, C 75.46, H 10.10; found, C 75.67, H 10.10

The *hydrochloride* separated from methanol ether as aggregates of micro platelets which sintered progressively above 235° and melted at 272–275° (uncorrected).

For analysis it was dried at 110° and 0.2 mm.

$C_{27}H_{43}O_2NCl$. Calculated, C 69.56, H 9.52; found, C 69.63, H 9.57

Δ^4 -*Dihydrojervone*—0.5 gm. of dried dihydrojervine was refluxed for 5.5 hours with 1.2 gm. of aluminum tert-butoxide in a mixture of 18 cc. of benzene and 9 cc. of acetone. At first almost clear, the mixture gradually showed considerable deposit which later became somewhat colored. After dilution and treatment with excess alkali, the mixture was extracted with chloroform. The washed and dried extract was concentrated and then repeatedly concentrated with benzene and, finally, *in vacuo*. However, the residue remained an oil. This was dissolved in 5 cc. of benzene and chromatographed at first with this solvent through 12 gm. of an active alumina. When material began to appear it was collected in 5 cc. portions. The first few fractions yielded an appreciable oily residue which smelled of acetone condensation products but from Fraction 5 yielded crystalline material. After Fraction 12 the solvent was changed to 0.5 per cent methanol in benzene to facilitate the otherwise very gradual elution. Following an interpretation of the general course of the elution and ease of crystallization, Fractions 26 to 38 inclusive were joined. On concentration *in vacuo* 0.28 gm. of a resin was obtained. On careful dilution of the acetone solution it gradually yielded a copious mass of needles which were collected with acetone-water (1:2). 0.195 gm. was obtained which melted at 122–128°. After recrystallization from dilute acetone it formed aggregates of needles which contained little solvent and melted sharply at 131–132°. It was readily soluble in the usual organic solvents.

$C_{27}H_{43}O_2N$. Calculated, C 76.18, H 9.24; found, C 76.62, H 9.37

From later fractions, especially after changing to 1 per cent methanol, a very small amount of unchanged dihydrojervine was recovered.

The *oxime* was prepared by refluxing the methanol solution of 28 mg. with 60 mg. of hydroxylamine hydrochloride and 0.2 gm. of sodium acetate. After 3 hours the concentrated solution was treated with excess ammonia and diluted before the sparingly soluble hydrochloride could separate.

The oxime separated as delicate needles. 24 mg. were collected. It tended to separate from methanol or diluted methanol as a gelatinous precipitate. From the warm diluted solution it crystallized as micro leaflets which lost birefringence at 293–297°.

$C_{27}H_{40}O_3N_2$. Calculated, C 73.58, H 9.15; found, C 73.30, H 8.98

N-Acetyl- β -dihydrojervinol Diacetate—0.1 gm. of β -dihydrojervinol was refluxed in 3 cc. of acetic anhydride for 3 hours. After removal of excess reagent the residue crystallized from methanol without solvent as micro platelets or needles which melted at 254–257°.

$C_{33}H_{48}O_6N$. Calculated, C 71.30, H 8.89; found, C 71.20, H 8.90

A suspension of 75 mg. of β -dihydrojervinol in 0.7 cc. of pyridine and 0.35 cc. of acetic anhydride was shaken at room temperature until dissolved and left for 20 hours. After repeated concentration *in vacuo* with benzene the residue was dissolved in methanol. From small volumes at 0° successive fractions of 28, 21, and 30 mg. were obtained. The first fraction (a) melted sharply at 256–257° while the second fraction (b) melted at 235–237°. The third fraction (c) melted again at 255–256°.

Found. (a) C 71.41, H 8.98

(b) " 71.51, " 9.06

(c) " 71.28, " 8.90

N-Acetyljervine Benzoate—0.1 gm. of *N*-acetyljervine (12) in 2 cc. of pyridine was treated in the cold with 0.1 cc. of benzoyl chloride. After 2 hours it was poured into excess cold dilute H_2SO_4 . After hardening, the product was collected. It formed rosettes of needles from 95 per cent ethanol which, although free from solvent, appeared to melt partly at 159–163° and then at 218–223°. When taken a few days later, it melted only at 219–223°.

$C_{38}H_{48}O_6N$. Calculated, C 75.61, H 7.94; found, C 75.58, H 7.71

All microanalytical work was performed by Mr. D. Rigakos of this laboratory.

SUMMARY

New observations with reinterpretation of previous data now permit of certain conclusions in regard to the structure of jervine. Most satisfactory is that of a 3(β)-hydroxy-11-keto- $\Delta^{5,8(9)}$ -steroidal secondary base in which the basic group is contained on the isoetyl side chain. The probable oxidic linkage appears also to be attached at least in part to the side chain.

There is evidence that the highly hydroxylated tertiary veratrine bases cevine, germine, and protoverine are $\Delta^{8(14)}$ -stenols and that their iso derivatives are Δ^{14} derivatives.

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TECHNIQUES FOR THE DEMONSTRATION BY CHROMATOGRAPHY OF NITROGENOUS LIPIDE CONSTITUENTS, SULFUR-CONTAINING AMINO ACIDS, AND REDUCING SUGARS*

By ERWIN CHARGAFF, CELIA LEVINE, AND CHARLOTTE GREEN

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, April 19, 1948)

Separation methods employing chromatography on filter paper, originally suggested for amino acids (1), are now being applied to the demonstration of a variety of substances in minute amounts. In the course of studies on the composition of certain tissue constituents, especially lipides and lipoproteins, which will be described at a later date, several techniques for the separation and demonstration of lipide and protein constituents were developed. These procedures may find a varied application and are, therefore, presented briefly at this time. The methods described here for the demonstration of choline and of reducing sugars will possibly be applicable to the quantitative microestimation of these substances.

EXPERIMENTAL

Nitrogenous Constituents of Phosphatides

The bases usually found in tissue phosphatides are choline, ethanolamine, and serine. The presence in certain lipide fractions of other free or substituted amines and amino acids, however, cannot be excluded in the light of previous studies presented from this laboratory (2). The separation experiments were carried out with test mixtures (containing 20 to 100 γ of the individual components per run, *i.e.* per 0.01 cc.) and with hydrolysates of tissue phosphatides, purified and freed of sphingosides as described previously (2). The phosphatides were hydrolyzed by boiling with 6 N hydrochloric acid for 24 hours and the chilled and filtered hydrolysis mixtures evaporated to dryness *in vacuo*. The solutions of the residues, which had been dried *in vacuo* over P_2O_5 and KOH, were adjusted to approximately pH 7 and contained about 1 mg. of amino N per cc. Portions of 0.01 cc. of the solutions were submitted to unidimensional chromatography on filter paper strips 50 cm. long (Schleicher and Schüll No. 597) for 12 to 18 hours. Some of the solvent mixtures employed and the R_F values (1) of the bases are summarized in Table I.

In all experiments, one set of paper strips served for the identification of

* This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

choline, and another was developed for primary amines. The presence of choline and its position on the paper were demonstrated by its conversion to choline phosphomolybdate, followed by the reduction of the latter to molybdenum blue; the primary amines were located by means of ninhydrin or by the appearance of fluorescent spots on the heated paper.¹

For the identification of choline (Solvents 2 to 4 in Table I), the papers were dried in air for 30 to 60 minutes and for the same length of time in an oven at 95°, sprayed with a 2 per cent aqueous solution of phosphomolybdic acid, washed by immersion in a large volume of *n*-butanol for 5 minutes and then for the same period in running tap water, and finally dipped into a freshly prepared 0.4 per cent solution of stannous chloride in 3 N HCl. Clearly circumscribed blue spots indicated the position of choline. No

TABLE I
Separation of Nitrogenous Phosphatide Constituents

Solvent No.	Solvent*	<i>R_F</i> values†		
		Serine	Choline	Ethanolamine
1	Phenol	0.35		0.43
2	<i>n</i> -Butanol-morpholine (3:1)	0.11	0.30	0.51
3	<i>n</i> -Butanol-dioxane (4:1)	0.14	0.26	0.27
4	<i>n</i> -Butanol-pyridine (4:1)	0.05	0.24	0.25

* The solvents, mixed in the volume proportions indicated, were saturated with water and the upper layers used for the experiments which were carried out at room temperature.

† Proportion of the distances of the starting point from the adsorbate and from the solvent front (1).

interference was noted by glucosamine, adenine, uric acid, or creatinine with butanol-morpholine as the solvent.

For the demonstration of serine and ethanolamine, the papers were dried in air (Solvent 2) or at 95° (Solvents 1, 3, 4) for 30 to 60 minutes, sprayed with a 0.1 per cent solution of ninhydrin in *n*-butanol, and then heated at 95° for 5 to 15 minutes, in order to develop the colored spots.²

¹ With respect to the fluorescence of amino acids and related substances, compare (3) and two brief notes (4, 5) which appeared after the completion of the experiments reported here.

² By the application of the method presented here, acid hydrolysates of the total phosphatide fraction of beef brain, purified as described before (2), were found to contain, in addition to ethanolamine, serine and choline, a hitherto unidentified constituent whose *R_F* value of 0.07 (in butanol-morpholine) placed it above serine. This substance, which is being investigated at present, gave the ninhydrin reaction. It was also found in a lipide preparation which, for further purification, had been precipitated from its saline suspension with acetone and subjected to prolonged dialysis.

Another procedure, particularly convenient when butanol-pyridine was employed as the solvent, consisted in the production from the adsorbates, by heating at 95° for 90 minutes, of spots showing a well defined fluorescence under the light of a quartz lamp.

Sulfur-Containing Amino Acids

The principle for the detection of these amino acids is their ability to catalyze the oxidation of sodium azide by iodine, $2\text{NaN}_3 + \text{I}_2 = 2\text{NaI} + 3\text{N}_2$ (6, 7). As little as 5 γ of cysteine or cystine and 20 to 25 γ of methionine could be demonstrated on filter paper by means of this reaction.

The paper strips were, following the separation, dried for 30 minutes at 95°, and sprayed with 0.05 N aqueous iodine containing 1.5 per cent of sodium azide. White spots on a light brown background indicated the position of the sulfur-containing amino acids. The contrast was sharpened considerably when the observations were made in ultraviolet light. Cysteine produced an almost immediate reduction of the iodine, cystine required about .15 minutes, and methionine up to 60 minutes.

When 0.01 cc. portions of test mixtures, containing (per cc.) 3 mg. each of cysteine and cystine and 6 mg. of methionine at pH 1, were subjected to separation with isobutyric acid (saturated with water) as the solvent, the following R_F values were found: cystine 0.26, cysteine 0.47, and methionine 0.70. Similar values were obtained with cystine and methionine when solutions containing only one of the amino acids were subjected to chromatography and also when ninhydrin was the developing agent (1). Cysteine, however, appeared to be decomposed and oxidized in part in the course of the chromatography. The chromatogram of a freshly prepared cysteine solution of pH 1 showed, upon treatment with ninhydrin, three zones of adsorption with R_F values of 0.18, 0.28, and 0.48. That the last two corresponded to cystine and cysteine respectively could be demonstrated by the use of the iodine-azide reaction which was immediate for the fastest, somewhat elongated component and required about 15 minutes for the middle adsorbate. The addition of a few KCN crystals appeared to diminish the oxidation of cysteine. When the cysteine solution was treated before chromatography with hydrogen peroxide in the arrangement of Dent (8), the cysteic acid produced gave no more iodine-azide test.

Preliminary experiments with human fibrinogen revealed two zones of iodine reduction, corresponding to cystine and methionine, when 0.01 cc. of a hydrolysate (corresponding to about 800 γ of the protein) was employed. This amount of protein contained, according to analyses in the literature (9), about 9 γ of cystine and 21 γ of methionine.

Reducing Sugars

In the course of a study of the sugars present in hydrolysates of purified cerebroside fractions from brain and spinal cord (10), a chromatographic

separation method similar to the one first described by Partridge (11) was employed. Since the demonstration of the separated reducing sugars by means of ammoniacal silver nitrate (11) often gave equivocal results, it was replaced by a different development method, namely, the production of brightly fluorescent spots by condensation of the reducing sugars with *m*-phenylenediamine. This reaction, briefly mentioned by Feigl ((7) p. 409), apparently is based on old observations on the formation of fluorescent substances from *m*-phenylenediamine and various aldehydes (12, 13).

The papers were, following separation, dried in air or for a short time at 105°, sprayed with a 0.2 M solution of *m*-phenylenediamine dihydrochloride in 76 per cent ethanol, and heated for 5 minutes at 105°. As little as 10 γ of the individual sugars produced well defined fluorescent spots when viewed under a quartz lamp. Arabinose, xylose, and ribose gave very strong orange-yellow fluorescence; fucose, rhamnose, glucose, galactose, mannose, fructose, and sorbose produced a yellow fluorescence of varying intensity. The reactions given by galacturonic acid, glucosamine, maltose, and lactose were weak, but discernible; the reaction of ascorbic acid was very faint. Polyhydroxy alcohols, such as sorbitol and inositol, did not react.

The solvents that could be used alone or in mixtures without impairment of the production of fluorescence were *n*-butanol, collidine, pyridine, and diacetone alcohol. Phenol, morpholine, diethylene glycol, and dioxane interfered with the reaction.

In the investigation of the carbohydrates present in the cerebroside of brain and spinal cord and in various lipid preparations isolated in the course of the fractionation of phrenosin and kersin (10), the principal attention was directed to the distinction between galactose, which is the normal sugar constituent of cerebroside, and glucose, found in the spleen cerebroside in Gaucher's disease (14). These two sugars run very near each other in most solvents (11). The solvent mixture which was found best suited for the separation and the development was butanol-pyridine. 1 part (by volume) of pyridine was mixed with 1.5 parts of water and 3 parts of *n*-butanol. To the upper layer which separated, 1 more part of pyridine was added and this mixture used for the chromatography. The R_F values of galactose and glucose were 0.33 and 0.37 respectively.

While the lipid studies are not yet concluded, the only sugar identified so far was galactose. No indication of the presence of appreciable amounts of glucose in preparations of phrenosin and kersin and related fractions could be found. This is in agreement with a very recent report on tissue cerebroside (15).

SUMMARY

Techniques are described for the demonstration, following chromatography on filter paper, of nitrogenous lipide constituents (choline, ethanolamine, serine), sulfur-containing amino acids (cysteine, cystine, methionine), and reducing sugars.

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CHEMISTRY OF THE CHICK EMBRYO

V. THE ACCUMULATION OF CYTOCHROME OXIDASE*

By MILTON LEVY AND NELSON F. YOUNG†

(From the Department of Chemistry, New York University College of Medicine,
New York)

(Received for publication, February 2, 1948)

The important position occupied by cytochrome oxidase in the present day concepts of oxidative metabolism led us to study its accumulation during the development of the chick embryo. The data of Albaum and coworkers (1, 2) describe the increase in cytochrome oxidase only in terms of total amount. No relevant data on weight or other quantities are given with which the amount of the oxidase could be related to the size of the actively growing organism at the time. The changes in metabolism and development proceed in regular sequence and adequate description of the whole sequence of development requires that correlation be carried as far as possible. This has been the general aim of this series of papers and the present report presents the results of the determinations of cytochrome oxidase on a large number of embryos whose ages range from 3 to 16 days of incubation.

Test System—Cytochrome oxidase activity has usually been estimated by measuring oxygen consumption in a system in which reduced cytochrome *c* is supplied in excess by the addition of a reducing substance such as *p*-phenylenediamine, hydroquinone, or ascorbic acid. The test system adopted for this study was the following mixture: 0.4 ml. of cytochrome *c* (0.6 mg.), 2.2×10^{-5} mole; 0.4 ml. of 2 per cent *p*-phenylenediamine; 1.0 ml. of a suspension of material to be tested; 0.2 ml. of *M* phosphate buffer, pH 7.3. The reaction was carried out at 37.2° in Barcroft-Warburg respirometers. The *p*-phenylenediamine was tipped in from the side arm after temperature equilibration. Oxygen consumption was measured for 1 hour. The cytochrome *c* was sufficient to insure maximal oxidative activity (10). With each determination a control vessel was also included which contained no added cytochrome *c*. The oxygen consumption of this control vessel was subtracted from that of the vessel containing cytochrome *c* and the difference was used as the measure of cytochrome oxidase activity.

The oxygen consumption in the control vessel is the sum of several factors: (1) basal respiration of the tissue, (2) autoxidation of *p*-phenylenediamine, (3) non-cytochrome oxidase catalysis which is due to cytochrome *b*

* Aided by a grant from the Ella Sachs Plotz Foundation.

† Present address, Sloan-Kettering Institute for Cancer Research, New York.

according to Stotz *et al.* (10), (4) oxygen consumption due to cytochrome *c* already present in the tissue. If the fourth of these factors is appreciable, then this vessel does not constitute a valid control for the estimation of oxidase activity. From the following considerations, it was concluded that the cytochrome *c* already present makes no significant contribution to the oxygen consumption in the control vessel.

1. Cytochrome *c* is very soluble and should be distributed throughout the aqueous phase of the system. If any were left in intact cells in a local high concentration or attached to "macromolecules" (9) in a particularly favorable position to react with the oxidase, then further grinding resulting in further dispersion of cytochrome *c* so attached should be reflected in decreased oxygen consumption in the control vessel. This effect was not observed even after long grinding in the homogenizers.

2. If we then assume that the cytochrome *c* is uniformly distributed and still contributes to the oxygen of the control, the addition of amounts of cytochrome *c* equal to that in the tissue should result in a measurable increase of oxidation. From the figures of Potter and DuBois (7) we find that the 10 day embryo contains 3.0 γ per gm. The addition of 0.3 γ of cytochrome *c* to a control vessel containing 100 mg. of tissue from a 10 day embryo had no effect on the oxygen uptake.

3. Potter and DuBois (7) reported at least a 6-fold increase in cytochrome *c* content from 6 to 12 days. During this time, it will be seen that a rise of only 50 per cent occurred in the control oxidation.

4. With concentrations of KCN reported by Stotz (10) to inhibit oxidase activity nearly completely, it was found that the control oxidation is inhibited only 25 per cent, while that of the vessels containing optimal amounts of cytochrome *c* is inhibited about 80 per cent. The more resistant system of the control vessels thus exhibits the same cyanide insensitivity as that reported for cytochrome *b*.

Embryological Material—Fertile eggs (from New Hampshire hens mated with Plymouth Rock cocks) collected in trap nests and received within 48 hours of laying were incubated at a temperature of 38.5° and at a relative humidity of 60 to 70 per cent. The eggs were rotated twice a day and removed in groups of three for analysis.

Preparation of Tissues—After removal from the incubator the embryos were freed of extraembryonic membranes, dried superficially, and weighed. They were then ground with 9 volumes of 1 per cent saline in glass homogenizers (8). Large embryos were minced in 9 volumes of saline in the Waring blender for 20 seconds before being ground in the homogenizers. Long treatment in the blender was found to inactivate the enzyme. 1 cc. portions (100 mg. of tissue) of these homogenates were pipetted into the Warburg vessels for the estimation of oxidase activity.

Results

Table I shows the effect of varying the amount of tissue in the test system used for this study. It demonstrates the strict proportionality of the amount of tissue to the oxidase activity. The lack of proportionality between the oxygen consumption of the control vessels and the amount of

TABLE I

Oxygen Consumption by 10 Day Embryo Tissue Plus 8 Mg. of p-Phenylenediamine

Tissue, mg.	50		100		150	
	0	2.2	0	2.2	0	2.2
	C.MM.	C.MM.	C.MM.	C.MM.	C.MM.	C.MM.
O ₂ used per hr.	30	132	51	258	69	370
Net O ₂ per 100 mg. tissue.		204		207		200

TABLE II

Accumulation of Cytochrome Oxidase in Chick Embryos

No. of embryos grouped	Average wet weight	Age calculated	Control, c.mm. O ₂ per 100 mg. per hr.	Oxidase activity, c.mm. O ₂ per 100 mg. per hr.
	mg.	days		
11	12.1	2.93	38	127
20	73.1	4.00	40	174
9	226	5.70	38	170
8	529	6.30	39	167
8	890	7.26	45	168
6	1,110	7.70	46	167
5	1,305	8.02	49	169
13	1,813	8.75	52	193
8	2,854	9.64	50	225
13	4,267	10.9	52	273.
12	7,035	12.0	62	284
12	7,814	12.7	62	316
10	10,560	13.9	67	324
10	13,680	14.8	66	334
11	16,230	16.0	68	345

tissue in each one reflects the varied natures of the factors contributing to the oxidation in these vessels.

A total of 157 embryos was used for this study. Individual determinations were made on each except for very young embryos, in which case two or more were pooled to provide enough tissue for a determination (2 × 100 mg.).

The whole series was arranged according to weight and divided into con-

venient groups according to size. The average age of the group was calculated from the average weight as recommended by Levy and Palmer (4). These data are included in Table II which shows the average oxidase activities as well as the control values for each group.

DISCUSSION

Various workers have determined the dry weight on chick embryos at various stages of development (6). Different breeds of chicks have shown

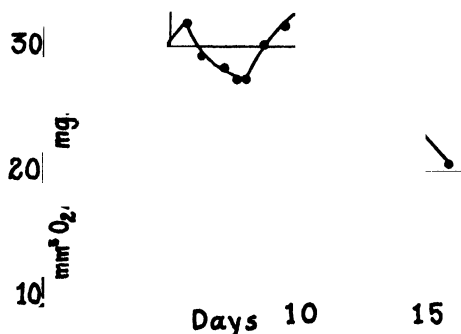


FIG. 1. Cytochrome oxidase activity per unit of dry weight against the calculated age in days.

TABLE III

Parameters of Phase Equations for Cytochrome Oxidase Accumulation

$\log \text{ c.mm. of O}_2 \text{ per mg. per hour} = i_Q + a_Q \log A.$

Interphase times, days	(3.0)*		.9	11.8	(16.0)*
$i_Q.$	-3.5†	-1.8	-3.3	-2.0	
$a_Q.$	6.3†	3.5	5.1	3.9	

* The figures in parentheses indicate the beginning and end of the period of experimental observations.

† The figures for this phase are based on two points only.

only minor differences in these values for a given age. Since Albaum and Worley (2) have indicated that the cytochrome oxidase activity of chick embryo tissue is proportional to the dry weight, it seemed of interest to plot the oxidase activity on a dry weight basis against time. Fig. 1 reveals that the oxidase activity is not proportional to the dry weight. The dry weight figures used in these calculations are those of Murray (5).

The description of growth data by appropriate equations relating the quantity measured, Q , to time, A , is a useful device. The equation which we have used to describe the accumulation of material during growth is log

$Q = i_Q + a_Q \log A$ (3). It was noted that at various times (called interphase times) it is necessary to make abrupt adjustment of the parameters i_Q and a_Q in order to fit the data. This is usual in all forms of growth equations covering development. A period in which the parameters remain unchanged is called a phase of growth in our scheme. A satisfactory modification of the equation is obtained by combining equations for the substance of interest with those for weight (W) (4). By plotting logarithms of concentration (Q/W) against logarithms of age, a relative accumulation diagram results, the slopes and intercepts of whose phases are simply related to i_Q and a_Q of the accumulation equations (4).

When the data of Table II are plotted in the way described above, three phases of accumulation are evident, with a fourth indicated by a single point. The interphase times and phase accumulation parameters are shown in Table III as obtained graphically. Each phase except the earliest is determined by at least four points falling on a straight line in the plot of the log concentration (c.mm. of O_2 per mg. per hour) of cytochrome oxidase against the log age calculated (in days). The interphase time at 4.3 days has appeared in all the entities measured so far in the present series; namely, wet weight, nitrogen, dipeptidase, and aminopeptidase (4). The interphase at 11.8 days is present in nitrogen (11.5 days) and aminopeptidase (11.9 days) accumulation diagrams. The significance of these times in terms of the metabolic and physiological activities of the embryo is not evident. The phase constants and interphase times are to be regarded at present as descriptive devices. The rapid rise in oxidase activity beginning at the 8th day was noted by Albaum and Worley (2).

SUMMARY

1. The cytochrome oxidase activity in chick embryos between the incubation ages of 3 and 16 days has been measured.
2. The data are used to construct accumulation diagrams and phases of constant multiplication rates in logarithmic time are demonstrated.
3. The cytochrome oxidase activity is not proportional to the dry weight.

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MIGRATION DURING HYDROLYSIS OF ESTERS OF GLYCEROPHOSPHORIC ACID

I. THE CHEMICAL HYDROLYSIS OF L- α -GLYCERYLPHOSPHORYL-CHOLINE*

BY ERICH BAER AND MORRIS KATES†

(From the Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada)

(Received for publication, April 5, 1948)

In view of the biological significance of the lecithins and of the fact that they have been known to the chemist for a hundred years (1), it is rather surprising that there should still remain doubts concerning the position of the phosphoric acid. Until recently it has been assumed by most workers that lecithins could be hydrolyzed chemically without causing a change in the position of the phosphoric acid and that the constitution and configuration of the resulting glycerophosphoric acid would be identical with that of the original phosphatide. Thus the presence of α - and β -glycerophosphoric acids (α -GPA and β -GPA) in the hydrolysis products of natural lecithins was interpreted as indicating the occurrence of both the α and β forms (2-4), and attempts were made to obtain these isomers in pure state (4-7). More recent evidence (8-10), however, although not fully conclusive, seems to be strongly in favor of the occurrence of a reversible phosphoric acid migration during the chemical hydrolysis of lecithins.

It was thought that an investigation of the acid and alkaline hydrolysis of L- α -glycerylphosphorylcholine (L- α -GPC), obtainable from autolyzed beef pancreas (11) and by synthesis (12), would provide information which might be useful in the elucidation of the reaction mechanism underlying the chemical hydrolysis of lecithins. The use of this pure enantiomer as substrate makes it possible to follow the course of the hydrolysis and especially the migration of the phosphoric acid group by physical (optical) as well as chemical means.

EXPERIMENTAL

Substrate—The L- α -glycerylphosphorylcholine used in this investigation was prepared synthetically as already described (12).

* An account of this work was presented before the meeting of the Canadian Physiological Society at London, Ontario, October 24-25, 1947.

† This paper forms part of a thesis which will be submitted by M. Kates to the Department of Chemistry, University of Toronto, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Acid Hydrolysis of L- α -Glycerolphosphorylcholine

Approximately 2.1 gm. of L- α -GPC ($[\alpha]_D = -2.9^\circ$) were dissolved in 10 ml. of distilled water; the solution was diluted with 12.5 ml. of 2 N hydrochloric acid and made up to a volume of 25 ml. The flask was transferred immediately to a water bath at $37^\circ (\pm 0.2^\circ)$ and the time noted. The concentration of the diester (total phosphorus determination) was 0.310 M and

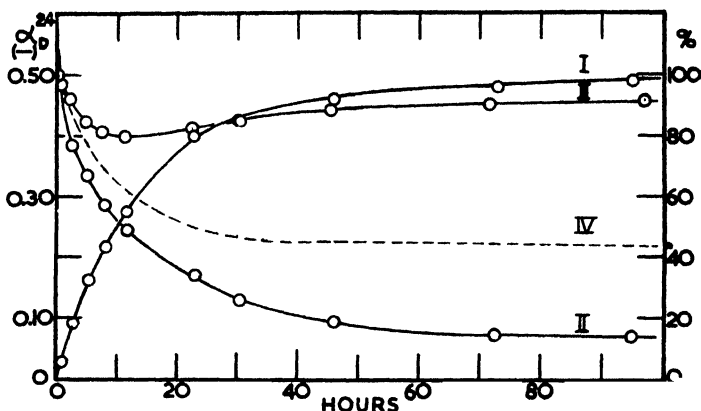


FIG. 1 0.310 M solution of L- α -glycerolphosphorylcholine in 1 N hydrochloric acid (pH 0.3) at $37^\circ (\pm 0.2)$. Curve I, hydrolysis expressed in terms of percentage of choline liberated; Curve II, observed changes in optical rotation, α_D (—), 24° , 2 dm. tube; Curve III, percentage of α -glycerol esters determined by periodic acid titration; Curve IV, calculated optical rotations which would be observed if L- α -GPC and L- α -GPA retain their full optical activity. The rotations were calculated by means of the following equation: calculated rotation = rotation(α -GPC) $\times$ (% α -GPC/100) + rotation(α -GPA) $\times$ (% α -GPA/100), where rotation(α -GPC) = -0.51° (rotation of the solution in a 2 dm. tube at 0 hour, obtained by the extrapolation of Curve II), per cent α -GPC = 100 minus per cent of liberated choline (obtained from Curve I), rotation(α -GPA) = -0.23° (rotation of a solution made up to resemble the final state of the hydrolysis solution, except that it contained pure L- α -GPA), and per cent α -GPA = per cent of liberated choline (obtained from Curve I) minus (100 minus per cent of α -glycerol ester (obtained from Curve III)).

the pH (Beckman pH meter) of the solution was 0.3. The changes during the hydrolysis were followed by observing the liberation of choline, the decrease in optical activity, and the variations in the α -glycerol ester content.

Liberation of Choline—At various time intervals a 1.00 ml. aliquot of the solution was withdrawn, run immediately into 25 ml. of water containing 1.0 ml. of 1 N sodium hydroxide, and the pH was adjusted to the Congo red end-point. The free choline was determined gravimetrically as reineckate. The course of the hydrolysis with regard to the liberation of choline is

shown graphically in Fig. 1, Curve I. The values for the reaction constant [$k = (2.303/t) \log (c_0/c)$] indicate a first order reaction; the k values at 48, 168, 318, 486, 696, 1368, and 1824 minutes were 1.22, 1.23, 1.25, 1.17, 1.15, 1.17, and 1.04×10^{-3} respectively. Up to 80 per cent hydrolysis the average value of k was 1.2×10^{-3} . The time required for the liberation of one-half of the choline was 10 hours. The hydrolysis was practically complete in 70 hours.

Optical Activity—At intervals, a portion of the solution was transferred to a 2 dm. tube and the rotation determined at room temperature (24–25°). The contents of the tube were returned to the original solution. The changes in rotation are shown in Fig. 1, Curve II.

Percentage of α -Glycerol Esters—At intervals a 1.00 ml. aliquot was withdrawn and diluted to a volume of 100.0 ml. The total α -glycerol ester content was determined on a 10 ml. portion of the diluted solution by titration with periodic acid according to Voris, Ellis, and Maynard (13). To insure that no errors due to dilution or concentration were involved, the total phosphorus was determined on each aliquot and these values were used for the calculation of the amount of α -GPC originally present. The periodate titration determines the sum of α -GPC and α -GPA present (see Fig. 1, Curve III). At the end of the hydrolysis the content of α -glycerol ester (α -GPA) reached a constant value of 91 ± 1 per cent.

To permit comparison with the hydrolysis products of natural lecithins, the glycerophosphoric acid mixture, obtained by acid hydrolysis of L- α -GPC, was isolated.

Isolation of Glycerophosphoric Acid Mixture As Barium Salts—A solution of approximately 1.7 gm. of the diester in 7 ml. of distilled water was diluted with 10 ml. of a 1.97 N hydrochloric acid and made up to a volume of 20 ml. The flask and contents were transferred to a water bath at 37° ($\pm 0.2^\circ$). The concentration of the diester (total phosphorus) was 0.302 M and the pH of the solution was 0.2. At the end of 110 hours at 37° the remainder of the solution (approximately 19 ml.) was freed from chloride ion by triturating with 2.3 gm. of silver oxide in the presence of 1 ml. of concentrated acetic acid, the excess silver ion was removed with hydrogen sulfide, and according to Folch's procedure (14), the glycerophosphoric acids were isolated in the form of barium salts. The barium salt mixture, after drying at 100° in a vacuum of 0.1 mm. of Hg over phosphorus pentoxide for a period of 2 hours, weighed 1.15 gm. (corresponding to a 75 per cent recovery of the glycerophosphoric acids) and the analysis proved it to be the barium salt of glycerophosphoric acid.

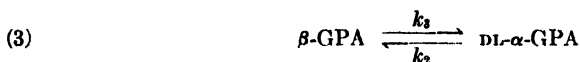
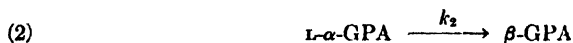
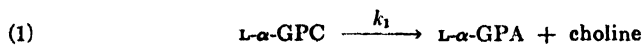
$C_2H_5O_6PBa$ (307.5). Calculated, P 10.08; found, P 10.00

The α -glycerophosphate content of the barium salt mixture was obtained

by titration with periodic acid (13). Prior to the titration, the barium ion was removed with sodium sulfate. After 1 hour, 0.0380 mm of glycerophosphate had consumed on the average 0.0333 mm of periodic acid, indicating the presence of 88 per cent of α -glycerophosphate. The optical rotation of the barium salt mixture in 2 N hydrochloric acid was $[\alpha]_D^{24} = -0.26^\circ$ (c , 10.0). A comparison of this rotation with that of pure L- α -barium glycerophosphate (15) in 2 N hydrochloric acid, $[\alpha]_D = -1.45^\circ$, revealed that 18 per cent of the total glycerophosphate was L- α -glycerophosphate. Thus the mixture of barium glycerophosphates obtained by acid hydrolysis of pure L- α -glycerylphosphorylcholine had a composition of 88 per cent α -glycerophosphate (18 per cent of L- α -glycerophosphate, 70 per cent of DL- α -glycerophosphate) and 12 per cent of β -glycerophosphate.

The information given in Curve I (free choline) and Curve III (content of α -glycerol ester) permits the calculation of the composition of the hydrolysis solution at any given time in terms of α -GPC, α -GPA, and β -GPA. Assuming that the α -GPC and α -GPA retain their full optical activity, the rotational changes to be expected under these circumstances can be calculated for the entire course of the hydrolysis (Fig. 1, Curve IV). A comparison of the hypothetical curve with the experimental curve (No. II) reveals that the observed optical activity decreases at a greater rate than would be expected if the loss were due only to an $\alpha \rightarrow \beta$ migration of the phosphoric acid. The progressively increasing loss in activity is a direct indication of the reversibility of this shift.

From the work of Bailly (9), Verkade, Stoppelenburg, and Cohen (16), and Chargaff (17), it is known that α -GPA in acid solution undergoes an intramolecular rearrangement, resulting in the formation of an equilibrium mixture of α - and β -GPA. It was therefore quite possible that the acid hydrolysis of L- α -GPA would follow a course expressed by Equations 1 to 3.



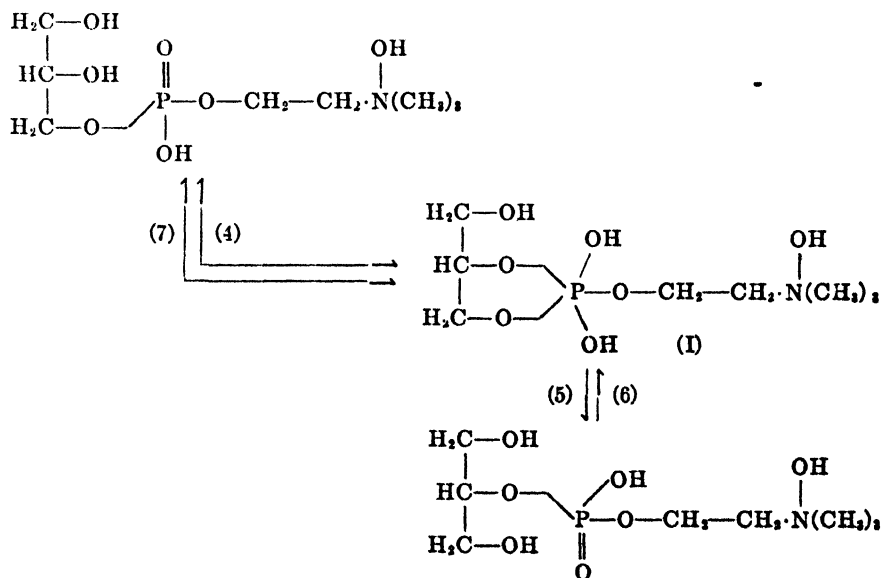
It was interesting to know whether this reaction mechanism was reconcilable with the changes observed on solutions of L- α -GPC in 1 N hydrochloric acid. The reaction constants¹ k_2 and k_3 were obtained by following the changes in optical activity (k_2) and content of α -glycerol ester ($k_2 + k_3$) of a 0.3 M solution of L- α -GPA in 1 N hydrochloric acid at 37° over a period of 90 hours. By means of the experimentally determined

¹ The experimental details will be reported elsewhere.

values of k_1 (1.2×10^{-3}), k_2 (2.5×10^{-4}), and k_3 (2.2×10^{-3}), the changes in optical activity and content of α -glycerol ester which should occur if Equations 1 to 3 do actually describe the acid hydrolysis of L - α -GPC were calculated. The observed rates at which the optical activity and the α -glycerol ester decreased were far greater than those predicted by calculation, indicating that Equations 1 to 3 are inadequate to describe fully the reaction mechanism underlying the hydrolysis of L - α -GPC. Although some of the phosphoric acid migration may occur in this way, the greater rate of change of the observed values suggests that the major part of the migration must take place while the choline ester is still intact, presumably via a cyclic ortho ester (I) as outlined in Equations 4 to 7 and in the accompanying scheme.

- (4) L - α -GPC $\rightleftharpoons$ cyclic ortho L -GPC
 (5) Cyclic ortho L -GPC $\rightarrow$ β -GPC
 (6) β -GPC $\rightarrow$ cyclic ortho DL -GPC
 (7) Cyclic ortho DL -GPC $\rightleftharpoons$ DL - α -GPC

The actual mechanism of the acid hydrolysis may be still further complicated by the fact that all substances in Equations 4 to 7 can, at least



theoretically, lose choline, forming the corresponding glycerophosphoric acids, which then equilibrate according to Equations 8 and 9.

- (8) L - α -GPA $\rightleftharpoons$ cyclic ortho L -GPA $\rightarrow$ β -GPA
 (9) DL - α -GPA $\rightleftharpoons$ cyclic ortho DL -GPA $\rightleftharpoons$

The present data are, however, insufficient to establish with certainty the mechanism, outlined by Equations 4 to 9, for the phosphoric acid migration.

Alkaline Hydrolysis of L- α -Glycerolphosphorylcholine

Approximately 2.3 gm. of the oily diester were dissolved in 10 ml. of water; the solution was diluted with 12.5 ml. of 2 N sodium hydroxide and made up to a volume of 25 ml. The flask was transferred immediately to a water bath at $37^\circ (\pm 0.2^\circ)$ and the time noted. The concentration of the diester (total phosphorus) was 0.336 M and the pH of the solution was 10.5.

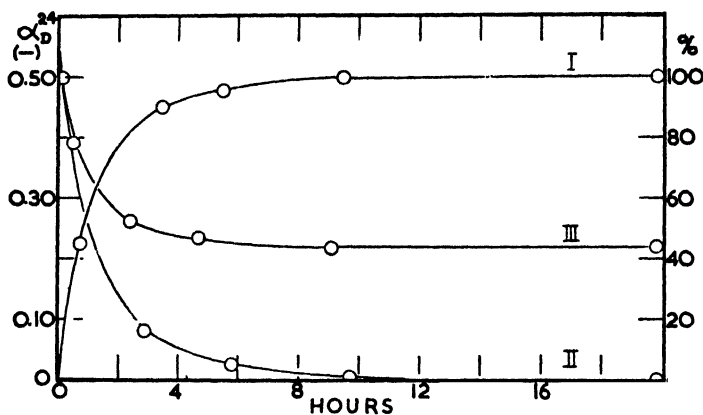


FIG. 2. 0.336 M solution of L- α -glycerolphosphorylcholine in 1 N sodium hydroxide (pH 10.5) at $37^\circ (\pm 0.2^\circ)$. Curve I, percentage of hydrolysis expressed in terms of choline liberated; Curve II observed changes in optical rotation, α_D^{24} , 2 dm. tube; Curve III, percentage of α -glycerol esters determined by periodic acid titration.

Liberation of Choline—At various intervals a 1.00 ml. aliquot was withdrawn and run into 25 ml. of water containing 1 ml. of 1 N hydrochloric acid and the free choline determined gravimetrically in the form of its reineckate (Fig. 2, Curve I). The rapid liberation of choline in alkaline solution made it difficult to obtain a sufficient number of points on the hydrolysis curve. However, the course of the liberation of choline in this medium approaches that of a first order reaction. With increasing time the reaction constant shows a distinct shift to lower values. At 42, 210, and 330 minutes, k was 1.42×10^{-2} , 1.16×10^{-2} , and 0.95×10^{-2} , respectively. The time required for the liberation of one-half of the choline was 0.9 hour. The hydrolysis is practically complete in 7 hours, a tenth of the time required for the acid hydrolysis of L- α -GPC.

Optical Activity—The measurements were carried out as described above. Since in alkaline medium the optical activity of L- α -GPA is immeasurably small, the observed changes in rotation are due entirely to the disappearance of L- α -GPC. For this reason the rate of change in rotation corresponds closely to that of the liberation of choline (see Fig. 2, Curve II). The specific reaction constants and half life period calculated from the optical values were $k = 1.2 \times 10^{-2}$ (30 minutes), 1.1×10^{-2} (174 minutes), and t_1 approximately 1 hour, respectively.

Percentage of α -Glycerol Esters—At various intervals a 1.00 ml. aliquot was withdrawn and run into a 100 ml. volumetric flask containing 1 ml. of 1 N hydrochloric acid in 80 ml. of water and made up to volume. The total phosphorus and the combined α -glycerol ester contents were determined as described above. Curve III in Fig. 2 presents graphically the changes in the total α -glycerol ester content. At the end of the hydrolysis the content of α -glycerol ester (α -GPA) reached a constant value of 44 ± 0.5 per cent.

Isolation of Glycerophosphoric Acid Mixture As Barium Salts—At the end of the 6th day the remainder of the solution (approximately 8.6 ml.) was neutralized to litmus with acetic acid and, according to Folch's procedure (14), the glycerophosphoric acids isolated as a barium salt mixture. The dried barium salt (0.1 mm., 100° , 2 hours over P_2O_5) weighed 0.80 gm., corresponding to a 90 per cent recovery of the glycerophosphoric acids, and upon analysis proved to be the barium salt of glycerophosphoric acid.

$C_3H_7O_6PBa$. Calculated, P 10.08; found, P 10.1

The α -glycerophosphoric acid content of the barium salt mixture was ascertained by periodic acid titration (13) after removal of the barium ion with sodium sulfate. At the end of 1 hour 0.0395 mm of glycerophosphate had consumed on the average 0.0160 mm of periodic acid, indicating the presence of 40.5 per cent of α -glycerophosphoric acid. The optical rotation of the barium salt mixture in 2 N hydrochloric acid was $[\alpha]_D = -0.29^\circ$ (c, 11.4), revealing that approximately 20 per cent of the total glycerophosphate consists of L- α -glycerophosphate. Thus the mixture of barium glycerophosphates, obtained by alkaline treatment of pure L- α -glycerylphosphorylcholine for 144 hours, has a composition of 20 per cent of L- α -glycerophosphate, 20 per cent of DL- α -glycerophosphate, and 60 per cent of β -glycerophosphate.

DISCUSSION

The investigation of the acid and alkaline hydrolysis of L- α -GPC has revealed that in both cases a reversible $\alpha \rightarrow \beta$ migration of phosphoric acid accompanies the liberation of choline, resulting in the formation of L- α -, DL- α -, and β -GPA. The equilibrium ratios of α - and β -GPA are a function

of the pH, the α form predominating on acid hydrolysis and the β form on alkaline hydrolysis. It is peculiar that the alkaline hydrolysis of methyl glycerophosphate (8), the acid treatment of β -glycerophosphate (9), the acid and alkaline hydrolysis of various components of brain cephalin (10), and the chemical hydrolysis of natural lecithins (2, 4) produce mixtures of α - and β -GPA similar in composition to those obtained by hydrolysis of L- α -GPC under comparable conditions; *i.e.*, predominance of the α form on acid hydrolysis and of the β form on alkaline hydrolysis, unless some common factor is involved.

The chemical hydrolysis of L- α -GPC yields the α -GPA in a partially racemized state due to the reversible phosphoric acid migration. It should be recalled that the α -GPA isolated from fermentation or glycolysis mixtures by Meyerhof and Kiessling (18) possessed the full optical activity of the pure synthetic L- α -GPA (15). The comparison was conducted on the dimethyl ether-dimethyl ester, which exhibits a considerably augmented optical rotation. Thus it is possible to isolate from natural sources α -GPA with full optical activity if undue exposure to acids, and particularly alkali, is avoided. In the case of α -GPA derived from natural lecithins (2), the most active preparations have had at best only 60 per cent of the activity of the optically pure α -GPA (15). It becomes highly significant that the optical activity of the α -GPA obtained by chemical hydrolysis of lecithins is considerably lower than that of the same material isolated from fermentation or glycolysis mixtures, especially since it is now established by the results presented in this paper that the chemical hydrolysis of L- α -GPC yields also a partially racemized α -GPA.

These observations suggest strongly that the chemical hydrolysis of lecithins is inevitably accompanied by a reversible phosphoric acid migration. Hence, the presence of α - and β -glycerophosphoric acid in the hydrolysates of lecithins cannot be considered any longer as an adequate proof of the simultaneous occurrence of α and β forms in the original lecithin.

Nevertheless, the natural occurrence of α -lecithins is firmly established. Karrer and Salomon's (2) observation that α -GPA, obtained from lecithins by alkaline hydrolysis, exhibits (as dimethyl ether-dimethyl ester) optical activity was the first entirely convincing demonstration of the occurrence of α -lecithins. Only an optically active α -lecithin can, on hydrolysis, yield optically active α -GPA, since the α -GPA formed from β -lecithins by acyl migration must be racemic. The more recent isolation of L- α -GPC from autolyzed beef pancreas (11), in which it is doubtless formed by enzymatic hydrolysis of α -lecithins, and the isolation of optically active dipalmitoyl lecithin from *Cysticercus fasciolaris* (19), from lung tissue (20), and from brain and spleen (21) are further evidence for the natural occurrence of α -lecithins.

On the other hand, if one recalls that the assumption of the natural occurrence of β -lecithins is based solely on the isolation of β -glycerophosphoric acid, the formation of which can also be accounted for by phosphoric acid migration during hydrolysis, one is forced to admit that at the present time conclusive evidence for the natural existence of β -lecithins is lacking.

It is obvious from the data presented in this paper that analytical procedures involving chemical hydrolysis cannot be relied upon to reveal the true constitution of lecithins or cephalins.

It is hoped that by studying the enzymatic hydrolysis of synthetic α -lecithins of known constitution and configuration analytical procedures may be developed which will establish unequivocally the constitution and configuration of the naturally occurring compounds. The synthesis of some of these lecithins has already been accomplished in this Laboratory, and that of others is in progress.

SUMMARY

1. The liberation of choline from α -glycerylphosphorylcholine in 1 N hydrochloric acid and in 1 N sodium hydroxide at 37° has been investigated. The reaction is of first order in both media. The time required for the liberation of one-half of the choline is, under the above conditions, 10 hours and 0.9 hour, respectively.

2. The removal of choline both in acid or alkaline medium is accompanied by a reversible $\alpha \rightarrow \beta$ phosphoric acid migration. At 37° a constant ratio of α - to β -glycerophosphoric acid is reached in 1 N hydrochloric acid after approximately 100 hours and in 1 N sodium hydroxide after 10 hours. The solutions in the equilibrium state contain 91 (± 1) per cent and 44 (± 0.5) per cent of α -glycerophosphoric acid, respectively.

3. A mechanism to account for the various changes observed during the hydrolysis of L- α -glycerylphosphorylcholine is suggested.

4. The occurrence of a reversible migration of phosphoric acid accompanying the chemical hydrolysis of glycerylphosphorylcholine, when considered with other evidence, leaves no doubt that a similar migration takes place during the chemical hydrolysis of lecithins. Analytical procedures involving chemical hydrolysis and investigation of the glycerophosphoric acid mixture cannot be relied upon to reveal the constitution of the glycerophosphoric acid moiety of the original lecithin.

5. There is conclusive evidence for the natural occurrence of α -lecithins. The existence of β -lecithins, however, is unsubstantiated. The β -glycerophosphoric acid, whose isolation was the only evidence suggesting the existence of β -lecithins, is, for the most part, an artifact.

The authors wish to express their gratitude to the Banting Research Foundation for grants to one of us (M. K.) during this investigation.

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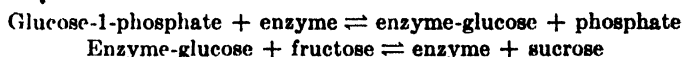
ON THE MECHANISM OF ACTION OF MUSCLE AND POTATO PHOSPHORYLASE

BY MILDRED COHN AND GERTY T. CORI

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis)

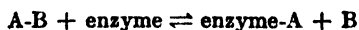
(Received for publication, April 15, 1948)

It has recently been reported by Doudoroff, Barker, and Hassid (1) that an exchange occurs between the phosphate group of glucose-1-phosphate and inorganic phosphate in the presence of sucrose phosphorylase and in the absence of acceptor. On the basis of this exchange, they postulate a mechanism involving the formation of an enzyme-glucose complex, thus endowing the enzyme with the ability to transfer glucose to fructose and other acceptors; *viz.*,



This mechanism has been confirmed by these authors by studying the reaction with arsenate (2). When glucose-1-phosphate, enzyme, and arsenate were mixed, again in the absence of acceptor, free glucose was formed. This could only have been due to the spontaneous decomposition of glucose-1-arsenate. Thus, the arsenate must have exchanged with the phosphate through the intermediate formation of an enzyme-glucose complex.

The proposed mechanism which might be described as an exchange of a bond between substrate and enzyme defines the nature of the substrate-enzyme interaction in contrast to the usual formulation which merely postulates the formation of an enzyme-substrate complex. This type of mechanism expressed in a generalized form, namely,



might well be applicable to other enzymatic reactions.

It seemed of interest to test the validity of this mechanism for the mode of action of muscle and potato phosphorylase and consider the possibility that A may be either glucose or phosphate. No exchange was found to occur between inorganic phosphate (labeled with P^{32}) and glucose-1-phosphate in the presence of muscle or potato phosphorylase when the addition of polysaccharide as acceptor was omitted. The absence of exchange was confirmed in the case of potato phosphorylase by the failure of arsenate to yield free glucose in a reaction mixture containing glucose-1-

phosphate but no acceptor. There was also no exchange of glucose (labeled with C^{14}) with glucose-1-phosphate during enzymatic synthesis of polysaccharide by muscle phosphorylase. The labeled glucose did not exchange with glycogen either in this system, which precluded a mechanism involving the transfer of a glucosidic bond through an interaction of enzyme and polysaccharide primer.

One further possibility was explored; namely, that an exchange might occur between adenylic acid which activates muscle phosphorylase *b* and inorganic phosphate. No exchange was observed. Thus the mechanism of enzyme action involving the type of bond transfer exhibited by sucrose phosphorylase is not apparent for the phosphorylases investigated in the present study and the extent of its applicability to other enzyme reactions awaits further investigation.

EXPERIMENTAL

Separation of Phosphates—The inorganic phosphate and glucose-1-phosphate in the reaction mixtures were separated as barium salts at pH 8.5 to 9. Inorganic phosphate was contained in the precipitate, glucose-1-phosphate in the supernatant fluid.

The precipitate was redissolved in dilute HCl and the precipitation at pH 8.5 to 9 was repeated. The resulting precipitate was dissolved, barium was removed with Na_2SO_4 , and the inorganic phosphate was then precipitated as the ammonium magnesium salt. After being washed with ammonium magnesium mixture, this fraction contained no organic phosphate.

To the soluble barium salts inorganic phosphate was added (in order to dilute contaminating inorganic P^{32}) and again removed by precipitation at pH 8.5 to 9. This was repeated several times. After removal of the barium the solution was found to contain besides glucose-1-phosphate a small amount of orthophosphate (1 to 8 per cent of the glucose-1-phosphate) as determined by the Fiske-Subbarow method (3). All values of radioactivity of glucose-1-phosphate listed in Tables I and II are corrected for the slight contamination which never exceeded 0.6 per cent of the orthophosphate radioactivity.

In experiments in which the phosphorylase reaction had gone to equilibrium, the recovery of glucose-1-phosphate could be improved by removal of the synthetic polysaccharide before fractionation with barium. Very little phosphate was removed with the polysaccharide when the latter was precipitated at pH 5 in 50 per cent ethanol. The supernatant fluid was then brought to pH 8.5 to 9 with $Ba(OH)_2$ and more ethanol was added to raise its concentration to 66 per cent. The resulting precipitate which contained inorganic phosphate and glucose-1-phosphate was separated into the two fractions as described above.

Exchange of Glucose-1-Phosphate and Inorganic Phosphate—It will be seen in Table I that in the aliquot to which starch had been added as a primer in the presence of potato phosphorylase the reaction attained equilibrium and that inorganic phosphate and glucose-1-phosphate had the same radioactivity (635 and 630 counts per minute per mm, respectively). Without addition of primer, however, no reaction took place and the glucose-1-phosphate showed no radioactivity. It will be noted from Table I that this was also the case in the experiment in which muscle phosphorylase served as catalyst. When a suboptimal amount of primer was added (12 mg. per cent of glycogen) the reaction was still far from equilibrium, but nevertheless the isolated glucose-1-phosphate had some radioactivity (47 counts per minute per mm), showing the extent to which the reverse

TABLE I
Exchange of Inorganic Phosphate (P^{32}) with Glucose-1-phosphate

Enzyme	Poly-saccharide	Reaction mixture				
		Time	Inorganic phosphate		Glucose-1-phosphate	
	mg. per cent	min.	micromoles per ml.	counts per min. per mm	micromoles per ml.	counts per min. per mm
Potato phosphorylase		Initial	5.2	2245	12.7	0
	None	30	5.2	2290	12.7	4
	125*	30	14.3	635	4.0	630
Crystalline muscle-phosphorylase <i>a</i>		Initial	12.2	2040	26.8	0
	None	20	12.1	2080	25.7	0
	12†	20	15.7	1500	21.4	47
	250†	20	25.4	745	11.0	

* Soluble starch.

† Glycogen.

reaction (glycogen + inorganic phosphate $\rightarrow$ glucose-1-phosphate) had taken place. With high concentration of primer (250 mg. per cent of glycogen) equilibrium was reached in the presence of muscle phosphorylase. In this experiment the polysaccharide was not removed before fractionation and the recovery of glucose-1-phosphate was so low that its radioactivity could not be measured.

Exchange of Glucose-1-Phosphate with Glucose—In the experiment summarized in Table II the phosphorylase reaction was allowed to go to equilibrium in the presence of glucose containing C^{14} . Glycogen was added to prime the reaction. After precipitation of the polysaccharide with 50 per cent ethanol, the phosphates were precipitated from the alcoholic fluid as barium salts and separated as already described. The isolated glucose-1-phosphate was found to contain no radioactivity. Glucose

remained in the 66 per cent alcoholic supernatant solution. Air was bubbled through the solution to remove the ethanol and the barium was precipitated with sulfuric acid. The amount of glucose in the solution was determined by the Nelson method (4). The glucose was found to have the same radioactivity in the experimental and control samples. No exchange with either glucose-1-phosphate or with glycogen could have taken place.

Exchange between Adenylic Acid and Inorganic or Glucose-1-Phosphate—Muscle phosphorylase *b*, which is active only when adenylic acid is added, was the enzyme used in this experiment as indicated in Table III. The reaction mixture contained initially 16 micromoles of glucose-1-phosphate

TABLE II

Exchange of Glucose (3755 Counts per Minute per Mg.) with Glucose-1-phosphate and Glycogen in Presence of Muscle Phosphorylase a

	Recovered glucose	Recovered glucose-1-phosphate
	counts per min. per mg.	counts p.r min. per mg
Control (no enzyme)	3805	0
Experimental	3835	0

TABLE III

Exchange of Inorganic Phosphate (P^{32}) with Adenylic Acid in Reaction of Glucose-1-phosphate and Glycogen in Presence of Muscle Phosphorylase b

	Inorganic phosphate	Ribose phosphate
	counts per min. per μ m	counts per min. per μ m
Control (no enzyme)	2090	0
Experimental.	1940	0

and 5.5 micromoles of adenylic acid per ml. The reaction was allowed to go to equilibrium in the presence of P^{32} (orthophosphate) and hence there was incorporation of P^{32} in glucose-1-phosphate. Then the reaction mixture was made 1.0 N with HCl and heated to 100° for 7 minutes. This led to hydrolysis of glucose-1-phosphate and left ribose phosphate (derived from adenylic acid) as the sole water-soluble barium phosphate. After isolation of the ribose phosphate by the procedure described for the isolation of glucose-1-phosphate, its ribose content (5) and phosphate content (3) were found to be in good agreement. There was no radioactivity found in the ribose phosphate. The radioactivity values of the inorganic phosphate given in Table III were measured after the hydrolysis of glucose-1-phosphate.

SUMMARY

No exchange occurred between inorganic phosphate and glucose-1-phosphate in the presence of muscle phosphorylase *a* or potato phosphorylase in the absence of polysaccharide as acceptor. Furthermore the phosphate of adenylic acid did not interchange in a complete reaction mixture containing inorganic phosphate (P^{32}), glucose-1-phosphate, glycogen, and muscle phosphorylase *b*. The possibility of an exchange between glucose (labeled with C^{14}) and glucose-1-phosphate was investigated in the presence of muscle phosphorylase and was found to be negative. The implications of these results are discussed in so far as they affect the mechanism of these enzyme reactions.

The authors wish to thank Dr. Barker and Dr. Hassid of the University of California for the radioactive glucose used in this work.

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ASSOCIATION BETWEEN RAPID GROWTH AND ELEVATED CELL CONCENTRATIONS OF AMINO ACIDS

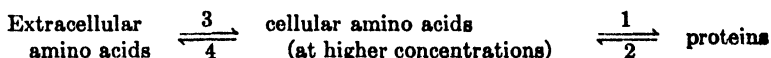
I. IN FETAL TISSUES

By HALVOR N. CHRISTENSEN AND JEAN A. STREICHER

(From the Children's Hospital and the Department of Biological Chemistry, Harvard Medical School, Boston)

(Received for publication, April 2, 1948)

Factors which accelerate growth or which produce wasting may do so by acting directly upon Reactions 1 and 2 in the following scheme:



In this case accelerated growth should tend to deplete the free amino acids of the tissues concerned. A good illustration is the precipitous fall produced in the glycine concentration of the liver when the conjugation of glycine is accelerated by feeding sodium benzoate (1). If, on the other hand, growth acceleration resulted from effects upon Reactions 3 and 4, there should be an associated elevation of the cellular concentrations of amino acids. Such an association has been observed in two instances which are reported in this and the accompanying communication (2).

In the present study a comparison has been made of certain amino acid concentrations in fetal and maternal plasma and tissues. The concentrations of both glycine and the non-glycine, non-glutamine ("residual") α -amino nitrogen of skeletal muscle were about 3 times as high in the fetus as in the maternal guinea pig. Similar relations were observed for both the cardiac and skeletal muscle of the rabbit. The higher fetal concentrations may be attributed to two factors: (1) concentration of amino acids by the placenta, and (2) greater concentrating activity of fetal muscle than of maternal muscle.

In the rabbit both factors were evident. In the guinea pig, concentration by the placenta appeared to account adequately for the higher concentrations of the fetal muscle; the fetal plasma was 4 to 6 times as rich in glycine and "residual" amino acids as the maternal plasma. After birth the plasma amino acid concentrations declined rapidly. In human fetal plasma, amino acids were observed to be 1.5 to 2 times as concentrated as in the maternal plasma.

Morse observed in 1917 (3) that the amino nitrogen (by the gasometric nitrous acid method) was higher in the plasma of the human umbilical cord than in the maternal plasma (averages, 8.7 and 6.4 mg. per cent).

Similar differences have been reported for rabbits (4) and dogs (5). The latter investigations, however, as well as others in human pregnancy (reviewed by Smith (6)), employed the unmodified Folin colorimetric method. There have been denials of such fetal-maternal plasma differences for both man and dog (7, 8). That these differences would be found also by the more specific manometric ninhydrin method was by no means certain, since the values of Morse and of others for amino nitrogen in the plasma of pregnant women are twice as large as those found by the ninhydrin method.

EXPERIMENTAL

The animals were in late pregnancy, the rabbits on the 28th day. The guinea pig fetuses were from 10 to 16 cm. in length. After fasting the animal for 12 hours, the abdomen was opened under ether anesthesia and blood was taken by needle from the umbilical veins (or in the case of several of the guinea pigs, by collecting blood flowing from the severed umbilical cord). Maternal blood was obtained by cardiac puncture. After killing the animals by a blow, the tissues were excised and analyzed for water, chloride, glutamine, glycine, and non-glutamine α -amino nitrogen, as previously described (9). The picric acid filtrates of plasma, however, were not subjected to treatment by lead acetate before analysis. The amino acid concentrations of the cellular water and the distribution ratios (cells to extracellular fluid) were calculated (9).

The human blood samples were obtained for us by Dr. Clement Smith and his associates of the Laboratory for Research on the Newborn, Boston Lying-In Hospital, at normal deliveries and at elective cesarean sections, from the antecubital vein of the mother and from the umbilical vein of the infant at approximately the same moment.

RESULTS AND DISCUSSION

The analytical results are presented in Tables I to III.

Three attributes were demonstrated for the guinea pig placenta (Table I) which have counterparts in the behavior of the cells of liver and muscle towards amino acids (1): (1) A single amino acid fed to the pregnant animal was concentrated by the placenta. (2) When high plasma concentrations of L-proline, L-histidine, or DL-methionine were produced by feeding each of these amino acids, the distribution ratio of glycine between fetal and maternal plasma was reduced, in some experiments almost to unity. (3) L-Glutamic acid occupied an unusual position in failing to show the second effect and in the extreme concentration which could be produced in the fetal circulation (37.9 mg. per cent of "residual" α -amino nitrogen, compared with 7.3 mg. per cent in the maternal plasma).

Concentration of amino acids by the placenta presents a very interesting

difference from concentration by tissue cells, in that here we are dealing with two extracellular fluids rather than a cellular and an extracellular fluid. There is no evidence that the amino acids in the fetal plasma are in a different form from those in the maternal plasma. Only fetal cells separate the two circulations in these three species, in the case of the rodents only a single layer of fetal endothelial cells (10, 11).

Both the glycine and the "residual" α -amino nitrogen of the plasma of pregnant guinea pigs were found to be lower than in plasma of non-pregnant

TABLE I

Distribution of Amino Acids between Maternal and Fetal Plasma and Muscle in Guinea Pig

The tissue concentrations are given in mg. of α -amino nitrogen per 100 gm. of cell water. The values in parentheses are ratios of the cellular to the extracellular concentrations. 20 mm per kilo of each amino acid were fed, two-thirds of the dose at the beginning and one-sixth at 1 and 2 hours. The animals were sacrificed at 3 hours.

Amino acid fed	Plasma						Muscle			
	Glycine			Residual			Glycine		Residual	
	Maternal	Fetal	Ratio, fetal to maternal	Maternal	Fetal	Ratio	Maternal	Fetal	Maternal	Fetal
Fasting (4)	0.47	2.40	5.3	1.84	8.7	4.7	4.9 (10)	16 (6.9)	14.9 (7.8)	41 (4.7)
Standard deviation	0.07	0.15	0.9	0.25	1.6	0.4	0.7 (1.8)	5 (1.4)	2.6 (0.9)	5 (0.9)
L-Glutamic	0.52	2.9	5.6	7.3	37.9	5.2	6.7 (12.2)	16.1 (5.6)	22.4 (2.9)	47 (1.2)
Glycine	7.8	9.9	1.3	1.05	6.2	5.9	26 (3.1)	24 (2.3)	12.5 (11.4)	39 (6.0)
L-Histidine	0.57	0.58	1.0	11.6	17.1	1.5				
DL-Methionine	0.28	0.53	1.9	5.53	10.2	1.9				
L-Proline	0.80	1.12	1.4	16.4	16.9	1.03	29 (3.4)	10.9 (9.2)	34 (2.0)	31 (1.7)

animals (1.8 mg. per cent for the "residual" α -amino nitrogen in pregnancy, 2.5 mg. per cent otherwise (1)). Bonsnes (12) reported reductions from the normal mean of about 4.2 mg. per cent to an average of 3.2 mg. per cent in plasma in human pregnancy. The low values were maintained throughout labor and until parturition. The diminutions probably result from the avid removal of amino acids by the placenta.

Two speculative suggestions upon the control of growth are pertinent. Robb (13) suggested that the constant relationship between the growth

rates of various tissues of the individual animal might be governed by the distribution among the tissues of some growth-promoting substance. He proposed that such a substance might be distributed according to characteristic partition coefficients, in analogy to the distribution of a solute among immiscible solvents. Twitty and van Wagtendonk (14) reported that an eye transplanted from a younger to an older larval *Ambystoma*

TABLE II

Distribution of Amino Acids between Maternal and Fetal Tissues in Rabbit

The concentrations are expressed in mg. per cent of α -amino nitrogen. The distribution ratio is the ratio of the cellular to the extracellular concentration.

Rabbit No.		Plasma		Skeletal muscle				Heart			
		Glycine	Residual	Glycine		Residual		Glycine		Residual	
				Cell concentration	Distribution ratio	Cell concentration	Distribution ratio	Cell concentration	Distribution ratio	Cell concentration	Distribution ratio
1	Maternal	0.8	4.7	5.8	6.9	19.4	4.0	1.1	1.4	26.0	5.3
	Fetal	0.70	7.0	9.7	13	54.8	7.4	4.7	6.4	67	9.1
	Fetal to maternal	0.9	1.5	1.7	1.9	2.8	1.9	4.3	4.6	2.6	1.7
2	Maternal	0.67	3.13	6.9	9.8	17.7	5.4	0.61	0.9	26.7	8.1
	Fetal	0.63	6.38	7.1	10.8	55.7	8.3	3.3	3.5	57.2	8.5
	Fetal to maternal	0.94	2.0	1.03	1.1	3.1	1.5	5.4	3.9	2.1	1.05

TABLE III

Distribution of Amino Acids between Maternal and Fetal Human Plasma

The concentrations are in mg. per cent of α -amino nitrogen and are followed by the standard deviations.

Type of delivery	Glycine			Residual		
	Maternal	Fetal	Fetal to maternal	Maternal	Fetal	Fetal to maternal
Normal (4 observations)	0.18	0.33	1.8	2.07	3.45	1.67
	± 0.02	± 0.02	± 0.1	± 0.10	± 0.37	± 0.10
Cesarean section (3 observations)	0.16	0.28	1.8	1.79	2.92	1.66
	± 0.01	± 0.01	± 0.1	± 0.23	± 0.63	± 0.39

tigrinum subsequently grew faster than its untransplanted mate remaining upon the younger salamander. Had transplanting the eye placed it in a richer medium? A larva 55 mm. long was found to have a whole blood amino nitrogen (by nitrous acid) of 188 mg. per cent; one 85 mm. long, 934 mg. per cent. Two conclusions were suggested: The transplanted eye

grew faster because it had been placed in a medium of higher amino acid concentration and the blood amino acid concentration increased with the age of the larvae as a result of the decline of growth potentialities of the organs. Unfortunately the nucleated red cells were not excluded from the blood samples, so that information was not obtained as to the concentration of amino nitrogen in the plasma or other extracellular fluids.

Our observations upon fetal muscle and upon regenerating liver support the view that increased protein synthesis and growth may be initiated or promoted by increased concentrations of amino acids. Normal cells, if they are not wasting, are necessarily maintaining each amino acid in concentrations adequate for building or replacement of many proteins. The concentrating function of cells for amino acids represents a possible point for the control of growth.

The authors are indebted to Dr. Clement Smith and Miss Ruth B. Cherry for obtaining blood samples for us at normal and cesarean deliveries, and to Miss Joan T. Rothwell for technical assistance.

SUMMARY

1. The concentrations of glycine and of non-glycine, non-glutamine amino acids were about 3 times as great in the skeletal muscles of fetal guinea pigs as in the maternal muscles. Similar relations were observed between the amino acid concentrations in the pregnant rabbit, for both skeletal and cardiac muscle. Amino acids were about 5 times as concentrated in the fetal plasma of the guinea pig as in the maternal plasma, in the rabbit about 1.5 to 2 times, in man 1.7 to 1.8 times. Skeletal and cardiac muscles of the fetal rabbit were able, despite their extremely rapid growth, to concentrate amino acids to a greater extent than did the maternal tissues.

2. Production of high blood concentrations of histidine, methionine, and proline by feeding these amino acids to pregnant guinea pigs greatly reduced the difference in the glycine concentrations of fetal and maternal plasma. After glycine was fed to the pregnant guinea pig, this amino acid appeared in higher concentrations in the fetal than in the maternal plasma. Feeding L-glutamic acid resulted in concentrations of over 40 mg. per cent of α -amino nitrogen in the fetal plasma, without any effect upon the glycine distribution.

3. The amino acid concentrations of plasma were decreased during pregnancy in guinea pigs as well as in human subjects.

4. The association of high cellular concentrations of amino acids with rapid growth has been discussed.

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ASSOCIATION BETWEEN RAPID GROWTH AND ELEVATED CELL CONCENTRATIONS OF AMINO ACIDS

II. IN REGENERATING LIVER AFTER PARTIAL HEPATECTOMY IN THE RAT

BY HALVOR N. CHRISTENSEN, JOAN T. ROTHWELL, ROBERT A. SEARS,
AND JEAN A. STREICHER

(From the Children's Hospital and the Department of Biological Chemistry, Harvard Medical School, Boston)

(Received for publication, April 2, 1948)

After removal of two-thirds of the liver of the rat, the remaining lobes grow so rapidly as to double their weight during the 2nd and 3rd days (1). This growth rate equals or exceeds that of tissue cultures, 8 day old chick embryos, and the fastest growing neoplasms (2). We have observed that, coincident with the period of most rapid growth, the liver cells contain elevated concentrations of amino acids (not including glutamine and glycine) and of glutathione

EXPERIMENTAL

Male albino rats, weighing 275 to 325 gm., from a highly inbred colony of the Sprague-Dawley strain were used. They were maintained on a stock diet of Purina laboratory chow. Anesthesia was produced by ether. The median and left lateral lobes of the liver were freed from connective tissue bands (mainly on the left margins and the anterior aspect) and a large loop of heavy silk thread (No. 00) was worked around and under both lobes; with the lobes lifted slightly, the thread was knotted tightly. Placing a clamp upon the pedicle and ligating it with fine thread in our hands often caused tearing of the vessels. Other details of surgery and postoperative maintenance were similar to those described by Crandall and Drabkin (3). The animals were not fasted before the operation; therefore the excised lobes were not suitable for control analyses. When the tissue analyses were to be made within 42 hours after hepatectomy, the rats were not fed in the interim, since food consumption was small and variable during the first 18 to 24 hours, and all animals were fasted for 20 hours before sacrifice. Animals allowed to live longer were supplied with the stock diet immediately after the operation.

One group of control rats was fasted 20 hours before study; another was subjected to a laparotomy which simulated the conditions of the hepatectomy without disturbing any lobes of the liver. These animals were then handled in the same way as the lobectomized animals.

The methods for preparing extracts of plasma, liver, and muscle and of analyses for water, chloride, glutamine, non-glutamine α -amino nitrogen, and glycine nitrogen have been described (4). In addition the picric acid extracts of liver were analyzed directly for α -amino nitrogen (by ninhydrin at pH 4.7) before removal of glutathione. The difference between this result and the α -amino nitrogen on the lead acetate filtrate provided an estimate of glutathione α -amino nitrogen. The measurement of the bound glycine, made in about half the cases, indicated that 79 to 83 per cent of the α -amino nitrogen precipitated by neutral lead acetate could be attributed to glutathione. (1 molecule of this peptide of course contributes 1 bound glycine molecule and 1 free α -amino nitrogen atom.) The concentration of each of the categories of α -amino nitrogen in the cell water of liver and muscle was calculated as usual on the basis of the water and chloride analyses (4).

Results

The analytical results are presented in Table I. For 20 hours after partial hepatectomy the amino acid concentrations of the remaining portion of liver were no higher than for controls subjected to a laparotomy. Between the 20th and 30th hours a rise to about 50 per cent above the control levels occurred in the concentration of "residual" amino acids of the liver cells. Coincident with this increase there were a proportionately smaller rise in the "residual" α -amino nitrogen of the plasma, an increase in the liver glutathione (whether indicated by the bound glycine or by the α -amino nitrogen precipitated by lead acetate), and a decrease in liver glutamine.

Amino Acid Conjugates Other Than Glutathione—Peptides other than glutathione were not increased in the regenerating lobes. Non-glutathione, conjugated α -amino nitrogen was calculated for dialysates of picric acid extracts of tissues as illustrated.

Total α -amino N after acid hydrolysis	74.6 mg. %
" " " before " "	44.1
Bound glycine " $\times$ 2	<u>27.6</u>
Sum	71.7
Non-glutathione, conjugated α -amino nitrogen	<u>2.9 mg. %</u>

Glutathione yields 1 glycine molecule and 2 additional α -amino nitrogen atoms upon hydrolysis. By correcting for the peptide nitrogen due to glutathione we could estimate other forms of conjugated α -amino nitrogen without removal of glutathione by lead acetate, which is apt to precipitate other peptides also (5). The resulting values for liver were 1.9, 2.9, and 0.3 mg., and for muscle 16.2 and 16.3 mg. per 100 gm. of fresh tissue.

Most of this conjugated α -amino nitrogen of muscle is probably due to the release of the amino group of histidine and methylhistidine upon hydrolysis of carnosine and anserine (6). About 10 mg., per 100 gm. of tissue, of undialyzable conjugated α -amino nitrogen were present in the picric acid filtrates of rat liver.

TABLE I

*Changes in Amino Acid Distribution Following Partial Hepatectomy in Rat**

The tissue concentrations are in mg. per 100 gm. of cell water. The values preceded by the $\pm$ sign are standard deviations. The values in parentheses are ratios of the cellular to the extracellular concentration.

	Plasma residual α -amino N	Liver			Muscle residual α -amino N
		Pb ppt.	Glutamine	Residual	
Controls (4)	4.32 ± 0.24	33 ± 3	22 ± 3	29.7 ± 2.0 (6.6 $\pm$ 0.8)	19.8 ± 1.1 (4.5 $\pm$ 0.6)
Controls, operated upon (4)	4.38 ± 0.21	32 ± 2.5	20 ± 3	35 ± 3 (8.0 $\pm$ 1.0)	19.1 ± 1.1 (4.3 $\pm$ 0.5)
Experimental, 18-20 hrs. (3)	4.04 ± 0.16	35 ± 3	10.8 ± 0.8	37 ± 3 (8.9 $\pm$ 1.0)	18.1 ± 0.7 (4.4 $\pm$ 0.1)
Experimental, 26-67 hrs. (10)	5.05 ± 0.47	40 ± 3	15.6 ± 5	47 ± 4 (8.9 $\pm$ 1.0)	19.0 ± 1.7 (3.7 $\pm$ 0.3)
Experimental, 90 hrs.	4.91	37	23	42 (8.2)	
Experimental, 200 hrs.	4.45	31	19	40 (8.5)	
Experimental, 400 hrs.	4.61	35	10	34 (7.0)	

* The following concentrations (for plasma, in mg. of α -amino nitrogen per 100 ml., and for tissues, in mg. per 100 gm. of cell water) were obtained for four fasting rats, and were not significantly changed by partial hepatectomy: plasma glutamine, 1.3 ± 0.1 ; plasma glycine, 0.51 ± 0.09 ; liver glycine, 7.6 ± 0.9 ; muscle glutamine, 5.4 ± 0.4 ; muscle glycine, 6.1 ± 0.5 .

Species Differences—A few species differences in amino acid distribution may be noted. Glycine made up a much smaller fraction of the amino acids of the liver, muscle, and plasma of the rat than of the guinea pig, and the concentration lacked the extreme variability observed in the latter species. In the cardiac muscle of the rat, glycine represented only 6 or 7 per cent of the non-glutamine amino nitrogen, whereas in the adult rabbit the heart contained a concentration of glycine scarcely higher than that of the plasma

(7). Glutamine made up about half the α -amino acid nitrogen of the heart in both the rat and rabbit, as in the dog (8). Glutamine was more concentrated in the liver than in the muscle of the rat; in the guinea pig the reverse was true. The plasma glutamine and residual α -amino nitrogen were about twice as high in the rat as in the guinea pig.

DISCUSSION

Increased amino nitrogen was observed by Vladimirova (9) in the regenerating limb of the amphibian axolotl. Presumably these analytical values include glutathione, which is elevated in the blastema and regenerated tissue (10).

There are several reasons for not attributing the observed rise of the amino acid concentrations in the liver simply to an inadequate deamination rate: (1) During the first 20 hours after lobectomy no increase occurred in the amino acids of plasma; in the liver the increases were not significantly greater than in controls operated upon. (2) The amino acid concentration of the liver increased proportionately more than that of the plasma, so that the distribution ratios (liver cells to extracellular fluid) were elevated. In contrast, whenever amino acids are entering the circulation faster than the liver can handle them, the distribution ratios are decreased (5). (3) Greatly accelerated deamination can be carried out with almost no increase in amino acid levels in the liver (5). (4) A lag in deamination fails to explain the coincident changes in glutamine and glutathione.

In our fasting animals hepatic restoration was occurring necessarily at the expense of other tissues, especially muscle. By what means was such a transfer of amino acids effected? A "flow" of the amino acids towards the liver might result from either an elevation of the distribution ratios of amino acids between liver and plasma or a depression of the distribution ratios between muscle and plasma. These factors appeared to participate about equally in the present instance (Table I).

Brues and Marble (11) showed that after removal of the median and left lateral lobes in the rat a latent period of 24 hours occurred, followed by a sudden rise of mitosis and cell multiplication to a maximum and then by a rapid decline during the succeeding days. Comparison of body and liver weights indicated that our animals responded very much like those of other investigators. The close association between the changes in amino acid concentration and in cell multiplication, with regard to latency, sudden rise, and subsequent decline, is emphasized.

The increase in the residual amino acid nitrogen of liver produced by laparotomy (Table I) is statistically significant, as is the increase in the distribution ratio between liver and plasma. In human subjects and experimental animals, surgical procedures, fractures, or infections produce a

strong acceleration of protein catabolism. At the same time the plasma amino acids are diminished (12). We are exploring the possibility that the decreased values for plasma result from an elevated concentration of amino acids by the liver.

SUMMARY

In rats subjected to partial hepatectomy, after a latent period and coincident with the period of most rapid hepatic restoration, the concentrations of amino acids in the liver cells were increased by about 50 per cent. Simultaneously glutathione was increased and glutamine decreased.

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STUDIES ON AMINO ACID EXCRETION IN MAN

I. AMINO ACIDS IN URINE*

By A. LEONARD SHEFFNER, JOSEPH B. KIRSNER,
AND WALTER L. PALMER

(From the Frank Billings Medical Clinic, Department of Medicine, University of Chicago, Chicago)

(Received for publication, February 25, 1948)

Recent interest in the rôle of proteins in gastrointestinal disease has prompted an investigation of certain aspects of this problem. Studies by Cannon and coworkers suggested to us that measurements of the intake and excretion of individual amino acids might supply more complete and significant data than the conventional nitrogen balances alone (1). Hence, in addition to the nitrogen balance, measurements were made of the intake (in food), level in the blood, and excretion in urine of eight amino acids; namely, leucine, isoleucine, valine, threonine, arginine, histidine, lysine, and methionine. The purpose of this paper is to describe the results of typical studies in two healthy, adult men on normal diets and also the adaptation of the microbiological technique utilized in this investigation for the amino acid analysis of urine. Studies on the excretion of amino acids in feces will be reported in a subsequent paper. The data presented in this report indicate (a) that the subjects of this study excreted in the urine a quantity of microbiologically available amino acids equal to 2.46 per cent of the amount ingested, and (b) that the renal tubules selectively reabsorb amino acids.

EXPERIMENTAL

Two healthy young men, students at the University of Chicago, were hospitalized in the metabolism section of the Albert Merritt Billings Hospital. Normal activity was permitted. In so far as could be determined by the medical history, a complete physical and laboratory examination, including tests of renal and hepatic function, x-rays of the lungs, gastric analysis, and basal metabolic rates, the subjects were free from physical defects. Subject 1 weighed 70 kilos; Subject 2 weighed 84 kilos. The diets contained approximately 1 gm. of protein per kilo of body weight per day, sufficient calories to prevent undue utilization of protein for energy, and vitamin

* Part of this report was presented before the Division of Biological Chemistry of the American Chemical Society at Chicago, April, 1948. The work described in this report was supported by grants from the Evaporated Milk Association and the American Dairy Association.

supplements. Each balance period was of 6 days duration and was preceded by a 3 day transition period, permitting adjustment to the constant diet.

Preparation of Food for Ingestion and Analysis—The food was prepared in the special diet kitchen of the metabolism section by dietitians specially trained in the dietary problems of metabolic research. The quality and quantity of the daily supply of food were kept as uniform as possible throughout the study. For analysis, a 30 to 40 per cent aliquot of a 1 day food sample (except pure carbohydrate and fat) was homogenized in a Waring blender with added distilled water. A second aliquot of the same diet was prepared and analyzed approximately 1 week later and the results of both measurements averaged.

For amino acid analysis, from 2.5 to 3 gm. samples of food homogenate were weighed accurately into a conical beaker and autoclaved in 20 ml. of 4 N hydrochloric acid for 10 hours at 120°. The samples were then boiled for 10 minutes with 250 mg. of norit A, filtered by suction, adjusted to pH 6.8, and made up to a volume of 1 liter. The concentration of the food sample was adjusted to contain approximately 0.025 mg. of nitrogen per ml.

Collection and Preparation of Urine Samples for Analysis—24 hour urine collections were stored under toluene in the refrigerator; the total collection for the 6 day metabolic period was combined and mixed. Aliquots were taken for total nitrogen, free ammonia, and for the assay of individual amino acids. The values for urea nitrogen and free ammonia will be included in a subsequent paper.

Total nitrogen was measured by a semimicro-Kjeldahl procedure, for which the digestion mixture of Campbell and Hanna (2) and the Pregl-Parnas-Wagner micro distillation apparatus were used. Urea was determined by the urease method. The individual amino acids were assayed microbiologically.

For amino acid analysis, 2.0 ml. of concentrated hydrochloric acid were added to 25.0 ml. of urine in a conical beaker. The beaker was capped with a small beaker and autoclaved for 10 hours at 120°. The acidified urine was then boiled 10 minutes with 250 mg. of norit A, filtered by suction, adjusted to pH 6.8, and diluted to volume. For the histidine assay, the original urine was diluted 1:40; for the other amino acid assays, it was diluted 1:4. All samples were hydrolyzed in duplicate and each hydrolysate analyzed at three different levels of concentration.

Several factors must be considered in preparing urine for microbiological assay. The urea must be eliminated, since it inhibits the growth of the assay organisms; this is accomplished by autoclaving in 1 N hydrochloric acid. Other interfering substances are also present in the urine; these are removed by further treatment of the samples with norit A, after autoclav-

ing with acid. The amino acid values are then distinctly higher and less erratic. A further advantage is the fact that the urine need not be greatly diluted. The assay for methionine continues to be erratic, however, when a synthetic medium is used, but yields consistent values when a semisynthetic medium containing oxidized peptone is employed.

Recovery experiments were performed for ten amino acids by adding measured amounts of pure amino acids to the urine and then subjecting the urine to the complete procedure. Only the L-amino acid was considered in calculating recoveries, since the organisms used were previously shown to be capable of utilizing only the natural isomer. The per cent of recovery varied from 90 to 110 per cent for eight of the amino acids analyzed. Since approximately 40 per cent of added phenylalanine and cystine was not recoverable, further studies of these two amino acids were not undertaken at this time.

Preparation of Plasma Samples—100 ml. of venous blood were drawn at the end of each period, transferred to a screw-capped bottle containing approximately 15 mg. of heparin, and thoroughly mixed. After centrifugation of the blood, the plasma was removed, and aliquots taken for nitrogen analysis, amino acid assay, and for the preparation of the tungstic acid filtrates.

For amino acid analysis of total plasma, 1.0 ml. of plasma was added to a conical beaker and autoclaved for 10 hours at 120° with 20 ml. of 2 N hydrochloric acid. The samples were then boiled for 10 minutes with 250 mg. of norit A, filtered by suction, adjusted to pH 6.8, and made up to 500 ml. Duplicate hydrolysates were prepared and each analyzed at three different levels of concentration.

The tungstic acid filtrate for determination of the free amino acids in plasma was prepared according to the method of Hier and Bergeim (3).

For determination of the total non-protein amino acid content of plasma, 20 ml. of tungstate filtrate were added to a conical beaker with 1.0 ml. of concentrated hydrochloric acid and autoclaved 10 hours at 120°. The sample was then adjusted to pH 6.8 and made up to 40 ml. If sediment appeared in the solution, the sample was filtered before analysis. As usual, duplicate hydrolysates were prepared and each one analyzed at three different levels of concentration.

Microbiological Procedure—The media employed are listed in Table I. *Streptococcus faecalis* was used with Medium 1 for the assay of leucine, isoleucine, valine, threonine, arginine, and histidine. *Leuconostoc mesenteroides* P-60 with Medium 2 was used for the assay of lysine and with Medium 3 for the estimation of methionine. The oxidized peptone was prepared according to the method of Lyman *et al.* (4).

The stock cultures of the assay organisms were maintained on a medium

TABLE I.

Media for Microbiological Assays of Amino Acids

The amino acid to be assayed is omitted from the medium. In each assay tube 2.5 ml. of medium were added to 2.5 ml. of the sample being analyzed.

	Medium 1	Medium 2	Medium 3
L-Asparagine	200 mg.	200 mg.	
L-Glutamic acid	200 "	200 "	
L-Arginine	200 "	200 "	
L-Histidine	200 "	200 "	
L-Lysine	200 "		200 mg.
L-Cystine	200 "	200 "	200 "
L-Proline	200 "	200 "	200 "
L-Tyrosine	200 "	200 "	200 "
DL-Alanine	200 "	200 "	
DL-Isoleucine	200 "	200 "	
DL-Leucine	200 "	200 "	
DL-Valine	200 "	200 "	
DL-Threonine	200 "	200 "	
DL-Methionine	200 "	200 "	
DL-Norleucine	200 "	200 "	
DL-Phenylalanine	200 "	200 "	
DL-Serine	200 "	200 "	200 mg.
DL-Tryptophan	200 "	200 "	200 "
Glycine	200 "	200 "	200 "
Oxidized peptone			5.0 gm.
Salts A			
KH_2PO_4	500 "	500 "	500 mg.
K_2HPO_4	500 "	500 "	500 "
Salts B			
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	200 "	200 "	200 "
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	10 "	10 "	10 "
NaCl	10 "	10 "	10 "
Thiamine chloride	500 γ	500 γ	500 γ
Pyridoxamine	1000 "	1000 "	1000 "
Calcium pantothenate	500 "	500 "	500 "
Riboflavin	500 "	500 "	500 "
Niacin	500 "	500 "	500 "
p-Aminobenzoic acid	500 "	500 "	500 "
Biotin	10 "	10 "	10 "
Folic acid (synthetic)	10 "	10 "	10 "
Adenine sulfate	10 mg.	10 mg.	10 mg.
Guanine	10 "	10 "	10 "
Uracil	10 "	10 "	10 "
Xanthine	10 "	10 "	10 "
Glucose	10 gm.	10 gm.	10 gm.
Sodium acetate		10 "	10 "
" citrate	20 "		
Adjust to pH 6.8			
Add distilled H_2O to	500 ml.	500 ml.	500 ml.

composed of glucose 1 per cent, Bacto-peptone 0.5 per cent, yeast extract 1 per cent, Salts A and B in the same concentrations as in Table I, sodium acetate 0.5 per cent, and agar 2 per cent. The stab cultures were kept in tubes covered with screw caps and stored in the refrigerator; capping the tubes with screw caps prevents the agar medium from drying and permits bimonthly subculturing.

After addition of standard or unknown solutions to the assay tubes, the volume was adjusted to 2.5 ml. with water, and 2.5 ml. of medium were then added to make the final volume 5.0 ml. Small metal caps were placed over each tube and the tubes were then autoclaved. The period of sterilization was measured from the time that the temperature within the autoclave reached 60°. After 10 minutes, during which time the temperature reached 110°, the steam was shut off and the autoclave allowed to cool till the chamber pressure descended to atmospheric pressure. In this manner caramelization is held to a minimum; yet growth by contaminating organisms is completely prevented. The tubes were then placed in a refrigerator and quickly cooled to room temperature. Prior to inoculation of the tubes, a culture was transferred to 10 ml. of liquid medium of the same composition as the medium described for maintaining stock cultures (agar omitted), and incubated 24 hours at 37°. The cells were centrifuged, washed once with sterile distilled water, recentrifuged, and suspended in 50 ml. of sterile distilled water. 1 drop of this suspension was added to each assay tube. To determine whether adding exactly the same number of organisms to each tube would give more consistent results, a sterile medium was inoculated with an assay organism and mixed well. Then exactly 2.5 ml. of the inoculated medium were added to each of a previously sterilized series of tubes containing 2.5 ml. of sample. This procedure, however, did not add to the consistency of the results. The dropwise inoculation of each tube was therefore adhered to because of its greater ease.

The inoculated tubes were incubated for 16 hours in a constant temperature water bath maintained at 35°.

Values were determined turbidimetrically with a Coleman junior spectrophotometer. A 19 × 105 mm. cuvette is a very convenient size in which to read the results. It is important in determining the minimum volume to be used in the tube that the light source does not pass through the meniscus of the solution. Therefore, by filling the upper half of the tube adapter slit with de Khotinsky cement, or a piece of wood painted black, the spread of the light source is diminished, and the minimum volume can be reduced to about 2.0 ml.¹ This size of tube is sufficiently wide to permit adequate deflection of the galvanometer by average turbidities developed in the

¹ Burfisher, W. C., Wilkens-Anderson Company, personal communication.

assay tubes to yield accurate readings. Turbidimetric measurement of the growth of the assay organisms reduces the time required for such an analysis and, in our hands, has yielded much more consistent results than have other procedures.

TABLE II

Microbiologically Available Amino Acids in Urine of Adult Men Fed Normal Diets
Averaged results of two 6 day periods for each subject.

Amino acid	Amino acids ingested per period		Amino acids excreted per period		Ingested amino acids excreted in urine	
	Subject 1	Subject 2	Subject 1	Subject 2	Subject 1	Subject 2
	gm	gm.	gm.	gm.	per cent	per cent
Leucine .	31.56	37.86	0.10	0.21	0.32	0.54
Isoleucine . . .	21.60	22.80	0.08	0.13	0.35	0.56
Valine .	21.30	24.60	0.10	0.15	0.47	0.61
Threonine .	15.00	19.44	0.27	0.45	1.77	2.31
Arginine .	19.08	28.02	0.12	0.19	0.61	0.68
Histidine .	10.80	13.08	0.72	2.44	6.66	18.63
Lysine . . .	18.72	28.80	0.31	0.89	1.66	3.07
Methionine	7.14	10.50	0.03	0.07	0.47	0.67

TABLE III

Concentration of Amino Acids in Plasma and Urine

Amino acid	Amino acids in hydrolyzed whole plasma		Free amino acids in plasma		Non-protein amino acids in plasma		Amino acids in urine		Renal clearance (C_r)*	
	Subject 1	Subject 2	Subject 1	Subject 2	Subject 1	Subject 2	Subject 1	Subject 2	Subject 1	Subject 2
	mg. per ml.	mg. per ml.	γ per ml.	γ per ml.	γ per ml.	γ per ml.	γ per ml.	γ per ml.		
Leucine	6.66	6.66	24.98	24.92	36.73	54.04	19.76	32.24	0.44	0.52
Isoleucine . . .	1.66	1.60	18.00	18.15	23.70	36.63	15.06	20.18	0.49	0.50
Valine	4.35	3.57	22.35	26.72	39.93	43.95	19.28	23.52	0.40	0.46
Threonine . . .	4.13	3.91	20.85	21.63	32.60	51.23	52.60	71.48	1.24	1.19
Arginine . . .	3.29	3.77	17.18	16.47	22.13	37.13	23.28	30.04	0.82	0.69
Histidine . . .	2.14	2.21	17.03	14.11	20.77	23.72	142.90	387.60	5.31	13.87
Lysine	6.41	8.41	20.78	24.70	36.10	60.21	61.75	140.54	1.32	2.00
Methionine . . .	0.46	0.50	3.23	4.52	3.20	4.83	6.66	10.92	1.59	1.94

* C_r = ml. of plasma cleared per minute = $U/P\sqrt{V}$, where U = concentration of an amino acid in urine, P = concentration of an amino acid in plasma, and V = volume of urine excreted per minute averaged for two 6 day periods.

RESULTS AND DISCUSSION

The data obtained for two representative periods in each subject are presented in Tables II and III. The quantity of amino acids excreted in the urine, averaged for the two subjects, ranged from 0.43 per cent of the amount

ingested for leucine to 12.65 per cent for histidine. For the eight amino acids studied an average quantity equal to 2.46 per cent of the amount ingested was excreted in the urine. These data should not be interpreted as indicating that the diet was the direct source of the amino acids present in the urine; they merely reflect the quantitative relationships. The fact that both subjects were in slightly positive nitrogen balance and maintained their weight throughout the period of study indicates that the diet was adequate and that the excreted amino acids were normal constituents of the urine. Comparison of the data in this paper concerning the excretion of amino acids in the urine of humans with that reported by other investigators is presented in Table IV. The agreement is good in every case.

TABLE IV

Comparison of Average Microbiological Values of Total Urinary Amino Acid Excretion of Human Subjects on Normal Diets

	Present report	Woodson <i>et al.</i> (5)	Dunn <i>et al.</i> (6)
	mg. per 24 hrs.	mg. per 24 hrs.	mg. per 24 hrs.
Leucine	25.8	21.2	31.2
Isoleucine	17.5	20.3	19.3
Valine	20.8	19.8	20.0
Threonine	60.0	53.8	57.8
Arginine	25.8	23.7	35.6
Histidine	263.3	203.3	188.5
Lysine	100.0	73.2	83.1
Methionine	8.3	8.6	11.9

Values were obtained for the concentration of free amino acids in plasma (tungstate filtrate) and the so called non-protein amino acids in plasma (tungstate filtrate hydrolysate), as well as of individual amino acids in hydrolyzed whole plasma. In all cases, with the exception of methionine, the hydrolysis of the plasma tungstate filtrate resulted in large increases in microbiologically available amino acid in the filtrate. The concentration of the eight amino acids in hydrolyzed whole plasma is presented for comparison with the concentration of non-protein amino acids in plasma in Table IV. Except for isoleucine, the general pattern of the non-protein amino acids, though not the actual values, conforms to that of the amino acids in whole plasma.

In the course of developing the procedure for the amino acid analysis of urine, it was found that, in those instances in which the urine could be analyzed without previous hydrolysis, hydrolysis resulted in an increase in the microbiologically available amino acids. If this increase were due to hydrolysis of polypeptides, or amino acid esters, it might be assumed that

amino acids in similar forms are also present in plasma. Therefore, the plasma tungstate filtrates were also hydrolyzed and the values thus obtained used in calculating the renal clearances of amino acids.

The increased amino acid values obtained by hydrolyzing the tungstate filtrates were attributed initially to hydrolysis of protein which had escaped precipitation by the deproteinization procedure. However, since the relative increases in the various amino acid values do not coincide with the amino acid concentration of total plasma proteins, plasma albumin, or plasma fibrin, as listed by Block and Bolling (7), it does not appear probable that the higher values for the tungstate filtrate are due to the hydrolysis of these plasma proteins. The increased values for several amino acids in the filtrate hydrolysates vary quite markedly over a period of several weeks and the percentage increase for each amino acid is independent of the increase of the other amino acids. It seems logical to assume, therefore, that the higher values are the result of hydrolysis of peptides, polypeptides, or esters of amino acids with compounds such as cholesterol.

The relative concentrations of non-protein amino acids and free amino acids of plasma tend to conform to the relative quantities of the individual amino acids ingested. The relative quantities of the amino acids excreted in the urine, however, do not follow this pattern. It is further observed that there is an enormous variation in relative renal clearance of the various amino acids. For example, the average renal clearance of histidine for the two subjects is 9.59 ml. of plasma cleared per minute. On the other hand, the average renal clearances of threonine, lysine, and methionine are between 1 and 2 ml., and for leucine, isoleucine, and valine less than 1 ml. of plasma cleared per minute. These data suggest that the renal tubules possess a marked selectivity in their normal reabsorptive function.

SUMMARY

The ingestion, blood levels, and excretion in urine of eight amino acids were measured microbiologically in two normal adult men on normal diets. The procedures employed for the assay of the eight amino acids are described.

The averaged quantity excreted in the urine ranged from 0.43 per cent of the intake for leucine to 12.65 per cent for histidine. For the eight amino acids studied, a quantity equal to 2.46 per cent of the amount ingested was excreted in the urine.

The normal renal clearance of the eight amino acids, averaged for the two subjects, varied from 0.43 for valine to 9.59 for histidine.

The authors wish to express their gratitude to Miss Blanche Parish, R. N., for supervising the collections and caring for the subjects, to Miss

Minnie Brandt for composing and preparing the diet, and to the subjects, Mr. John Doull and Mr. Richard Herz, for their cooperation during the study.

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THE UTILIZATION OF LABELED GLYCINE IN THE PROCESS OF AMINO ACID INCORPORATION BY THE PROTEIN OF LIVER HOMOGENATE*

BY THEODORE WINNICK, FELIX FRIEDBERG,†
AND DAVID M. GREENBERG

(From the Division of Biochemistry, University of California Medical School, Berkeley)

(Received for publication, February 17, 1948)

The incubation of rat liver slices in an oxygenated medium in the presence of either S³⁵-labeled methionine (1) or C¹⁴-labeled alanine (2) results in an uptake of the radioactive amino acid into the protein of the slices. A similar reaction has been noted between the protein of intestinal slices and C¹⁴-labeled glycine (glycine*) (3). Presumably the residues of the labeled amino acids become components of the protein molecules. The inhibition of the C¹⁴ incorporation by heat or sodium azide suggests that the process is mediated by enzymes (3).

Recently (4) it was reported that glycine* is incorporated into the proteins of homogenized preparations of spleen and liver upon incubation under suitable conditions. Further work has shown that the process involved is not simply one of glycine* incorporation into protein. Instead, the major portion of the C¹⁴ found in the homogenate protein can be attributed to other amino acids, particularly serine, derived apparently from glycine. These results are reported separately (5).

The present paper describes the effect of various metabolites, inhibitors, and certain experimental conditions on the process of C¹⁴ incorporation by liver homogenate protein in the presence of glycine*.

Of several different organs tested, rat spleen yielded the most active homogenate preparations. However, spleen tissue is complex histologically, in that it consists of several types of small cells, and the destruction of the latter by mechanical grinding is difficult to gage. By contrast, liver consists mainly of cells of one type. These cells are rather large (about 25 to 30 μ in diameter in the rat) and are readily ruptured in a glass homogenizer. The small fraction of the hepatic cells which escapes disintegration can be removed by low speed centrifugation. Homogenates prepared in this manner are free from intact liver cells. They contain abundant nuclei, as well as cytoplasmic material, leucocytes, and erythrocytes.

* Aided by grants from the American Cancer Society (recommended by the Committee on Growth of the National Research Council) and the National Cancer Institute, United States Public Health Service.

† National Cancer Institute Senior Research Fellow.

In the presence of glycine* these cell-free preparations can incorporate C^{14} into their protein substance at rates comparable to those observed with liver slices.

As in *p*-aminohippuric acid (PAH) synthesis (6), the enzyme activity is found to be associated with the insoluble particles of the rat liver cells. However, as might be expected, PAH synthesis (from glycine and hippuric acid) and glycine* utilization by homogenate protein differ markedly in certain respects. One difference which may be mentioned at this point is that PAH synthesis was observed only in liver and kidney preparations, while glycine* utilization occurs in homogenates of virtually all of the several organs.

TABLE I

Composition of Standard Medium

The solution was prepared with sterile distilled water and saturated with a mixture of 95 per cent oxygen-5 per cent CO_2 gas. Its final pH was 7.4. It was found advisable to prepare fresh solutions about twice monthly and to store these in the refrigerator to prevent spoilage.

Constituent	Concentration
	gm. per 100 ml. solution
NaCl..	0.72
NaHCO ₃	0.25
KH ₂ PO ₄	0.10
MgSO ₄ ·7H ₂ O.	0.06
Glucose.....	0.10
Sodium citrate...	0.03

EXPERIMENTAL

Radioactive Glycine—The glycine* employed was labeled with C^{14} on the methyl carbon position (7). It had a specific activity of 3.1 microcuries (approximately 400,000 counts per minute per mg., as measured with the mica window counter tube employed in these experiments). Sterile solutions containing 1 mg. of glycine* per ml. of distilled water were prepared and stored in the refrigerator. Aliquots, generally containing 0.2 mg. (80,000 counts), were used for individual experiments.

Standard Medium—A modified Krebs-Henseleit medium, of the composition given in Table I, was used in the preparation of the liver homogenates. 3 ml. of 1 per cent calcium chloride were added to 100 ml. of medium just prior to use.¹

Preparation of Homogenate—Rats (150 to 200 gm.) were killed and the

¹ A precipitate or turbidity due to calcium carbonate develops slowly on standing.

livers removed. 1 gm. portions of liver were homogenized thoroughly with 15 ml. quantities of cold standard medium in a glass homogenizer equipped with a close fitting, rapidly revolving piston. The resulting suspension was then centrifuged for 2 minutes at 500 to 600 R.P.M. (in an angle head centrifuge). The supernatant liquid was removed by siphoning and employed in the experiments to be described. The small sediment of surviving liver cells was discarded. The pH of the homogenate was 7.5 to 7.6.

Preparation of Washed Insoluble Cell Particles—Liver homogenate was prepared with either the standard medium¹ or an isotonic saline-bicarbonate solution,² in the manner already described. About 200 ml. of this homogenate were centrifuged for 15 minutes at 4000 R.P.M. in a refrigerated (angle head) centrifuge. The supernatant solution was discarded. The sediment³ was mixed thoroughly with 75 ml. of standard medium, or saline-bicarbonate solution, and centrifuged as before. This last step was repeated a specified number of times. Finally, the sediment was suspended in 10 ml. of either standard medium or saline-bicarbonate and used without delay in the incubation procedure.

*Incubation of Homogenates in Presence of Glycine**—3 ml. aliquots⁴ of freshly prepared homogenate were pipetted into 50 ml. glass-stoppered flasks containing measured amounts of glycine. Each flask was flushed thoroughly with 95 per cent oxygen-5 per cent carbon dioxide mixture, stoppered, and then agitated in a 37° water bath for a definite time, generally 90 minutes.

In certain specified cases, 0.5 ml. aliquots⁴ of the preparation of washed insoluble particles were used instead of 3 ml. of whole homogenate. In these instances, 2.5 ml. of nutrient medium were added to each flask.

Separation of Radioactive Protein—At the conclusion of the reaction period, the protein of the homogenates was precipitated by the addition of 10 ml. of 12 per cent trichloroacetic acid to each flask. The precipitates were collected by centrifugation, washed three times with 12 ml. quantities of 5 per cent trichloroacetic acid, and then twice with acetone, being centrifuged after each washing.

Measurement of Radioactivity of Protein—The protein material in each tube was suspended in acetone and transferred to a weighed 5.5 cm. No. 50 Whatman filter paper, with the aid of a Tarver type filter (8). The precipitates (12.5 cm. in area, generally 22 to 26 mg. in weight) were dried for

¹ Composed of 8.0 gm. of NaCl and 2.5 gm. of NaHCO₃ per liter of water, saturated with 95 per cent O₂-5 per cent CO₂ gas; pH approximately 7.3.

² It consisted of two layers: a light brown upper layer, rich in nuclei and mitochondria, and a smaller bottom layer of erythrocytes. It was not considered important to separate these layers.

⁴ Each contained about 25 mg. of protein.

an hour at 100° and then equilibrated in air.⁵ The papers were reweighed, and the radioactivity of the layers determined with the Autoscaler⁶ model Geiger-Müller counter.

In order to compensate for the self-absorption of radiation, the radioactivity of each protein sample was compared to that of a glycine standard, prepared by diluting a definite amount of glycine* with a quantity of inactive organic material (glycine) of the same weight and layer thickness as the protein. In this way the concentration of labeled carbon in the protein could be ascertained. This concentration was generally expressed as counts of C¹⁴ per minute per mg. of protein per hour of incubation.

TABLE II

Effect of Repeated Washing with Standard Medium on Activity of Insoluble Homogenate Particles

The results were corrected for a background of 51 counts per minute. Errors due to self-absorption of radiation were compensated by comparison with glycine* standards, as indicated in the experimental section. The triplicate values in the second column pertain to separate incubations of aliquots taken from the homogenate preparation at different stages. The quantity of protein in the aliquots was in the range of 20 to 30 mg.

Experiment	Radioactivity of protein, counts per min. per mg. per hr. of incubation	Average per cent de- viation of individual results from mean value
Original homogenate	7.0, 7.1, 8.4	7.5
Homogenate centrifuged 15 min. at 4000 R.P.M.; supernatant assayed	1.2, 1.4, 1.7	12.6
Sediment from preceding experiment assayed	14.7, 15.9, 16.3	4.3
Sediment washed once	12.3, 13.7, 14.6	6.3
“ “ 3 times	11.1, 11.4, 11.7	1.8
“ “ 5 “	8.1, 9.1, 9.5	6.5

Accuracy of Radioactivity Measurements and Reproducibility of Results—

The protein samples usually had activities of the order of 5 to 10 times the background strength. They were counted for time intervals (generally 5 to 20 minutes) sufficient to reduce the counting error to less than 3 per cent.

All experiments were performed at least in duplicate. Table II illustrates the degree of reproducibility of replicate assays. In Tables III and IV the averages of duplicate determinations are given. The average agreement between these duplicate determinations was 6 per cent.

Inasmuch as homogenates prepared on different days (from different

* These preparations contained 14 per cent nitrogen.

⁶ Manufactured by Tracerlab, Inc., 55 Oliver Street, Boston.

livers) sometimes differed as much as 30 per cent in activity, it was simplest to express certain of the results (Tables III and IV) in terms of relative, rather than absolute, radioactivity units. In these instances, values are given for the radioactivities of the reference homogenates.

RESULTS AND DISCUSSION

Rate of C^{14} Incorporation into Protein—Curve 2 of Fig. 1 indicates that the quantity of C^{14} incorporated into protein increased uniformly with time. From the initial slope of the curve, it may be calculated that 0.004 γ of labeled carbon was contained in each mg. of protein after 1 hour. 1 γ of this carbon was equivalent to 2000 counts per minute. The radioactivity

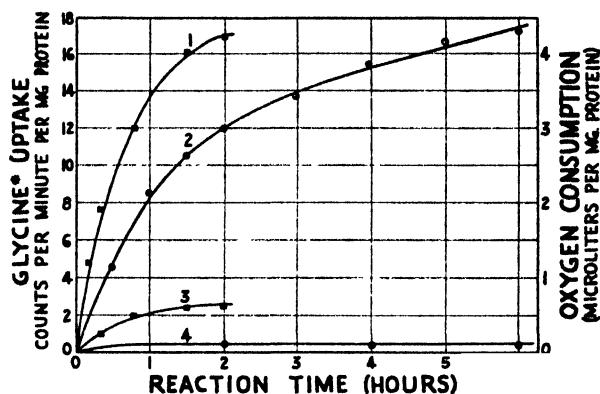


FIG. 1. Rates of oxygen consumption and C^{14} utilization by liver homogenates. Each of a series of flasks contained 0.2 mg. of glycine* and 3 ml. of homogenate. Curves 1 and 3, oxygen consumption by active and boiled homogenates; Curves 2 and 4, C^{14} incorporation by active and boiled homogenates. The oxygen consumptions were measured in the Warburg apparatus in the presence of ordinary glycine and in an air atmosphere

incorporated into the 25 mg. of protein of the average homogenate in this period was equivalent to 0.1 γ of labeled carbon, or 0.25 per cent of the total amount employed (40 γ of carbon in 0.2 mg. of glycine*).

The heated homogenates (Curve 4) exhibited virtually zero activity.

The rate of C^{14} uptake by homogenates was approximately one-third that found with liver slices under almost identical conditions.⁷

It is of interest that the homogenate utilized oxygen (Curve 1). The initial rate of oxygen consumption was approximately one-fourth that with liver slices.⁷ Heated homogenate showed a very low consumption (Curve 3). That the oxygen consumption was related to the glycine* utilization

⁷ The reaction rate with slices was determined by Mr. P. Siekevitz of our department.

by protein was further indicated by the observation that the latter process was drastically inhibited when the incubations were conducted in a nitrogen atmosphere. Frantz and coworkers (2) found that liver slices do not take up labeled alanine under anaerobic conditions.

Homogenates, evidently, afford particularly favorable conditions for the growth of microorganisms. In some preliminary experiments, in which undue contamination apparently occurred, Curve 2 of Fig. 1 assumed an autocatalytic character, with a steep upward inflection after 3 to 5 hours.

Influence of Glycine Concentration*—Fig. 2 indicates that the absolute amounts of C^{14} incorporated into protein increased with increasing concentration of glycine* in the homogenate system. However, the efficiency of

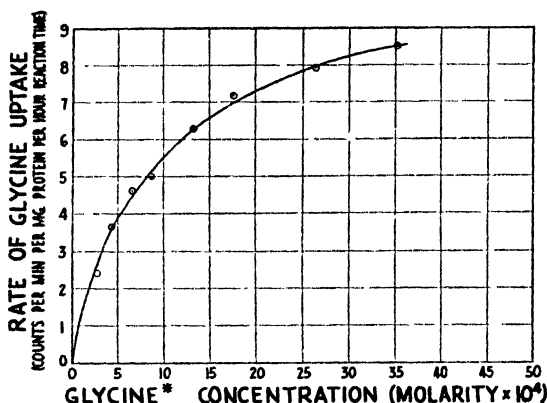


FIG. 2. Effect of variations in glycine* concentration on the rate of C^{14} incorporation into the protein of homogenate. The reaction time was 90 minutes. Each of a series of flasks contained 3 ml. of homogenate and a quantity of glycine* ranging from 0.07 to 0.85 mg.

the process was greater at lower concentrations. For example, at a glycine* concentration of 2×10^{-4} M, about 0.3 per cent of the total C^{14} in the system was taken up by the protein per hour, while at a concentration of 35×10^{-4} M, the corresponding percentage was 0.06. Similar results were obtained by Melchior and Tarver (1) with liver slices and labeled methionine.

Effect of pH—The preparation of washed insoluble particles (rather than the whole homogenate) was employed here to facilitate pH adjustments. The rate of glycine* utilization was found to have a wide optimum pH range, centering about pH 7.5 (Fig. 3). By contrast, the curve for PAH synthesis exhibited a very sharp peak at pH 7.5 and relatively low activity above pH 7.9 (9).

Association of Protein Activity with Insoluble Particles of Homogenate—

The enzyme system which regulated the C^{14} incorporation into protein was found to be associated with the insoluble particles of the homogenate, since it sedimented together with these particles (Table II). The particles were about twice as active (per mg. of protein) as the whole homogenate, or about

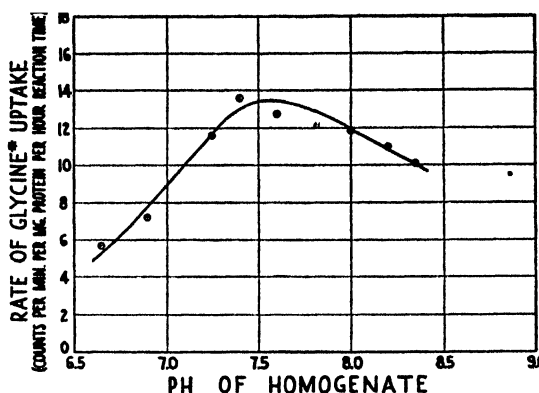


FIG. 3. Relation between pH and rate of incorporation of C^{14} into protein. 0.5 ml. aliquots of a preparation of washed insoluble particles of liver homogenate were added to 2.5 ml. portions of nutrient solution containing varying amounts of $NaHCO_3$. The pH values were measured at the conclusion of a 90 minute reaction period. 0.2 mg. of glycine* was used per flask.

TABLE III

Effect of Different Preliminary Treatments on Activity of Liver Homogenates

Experiment	Relative radioactivity of protein
Liver homogenized with standard medium	100†
“ “ “ distilled water; medium added subsequently	19
Liver frozen (with dry ice) and thawed 6 times prior to being homogenized	
Liver homogenate lyophilized and reconstituted with water	14
Homogenate maintained for 24 hrs. at 3° prior to addition of glycine* and incubation	18
Liver perfused <i>in situ</i> with saline prior to being homogenized	104

† This activity averaged 8.2 counts per minute per mg. of protein per hour of reaction time with homogenates prepared on different days.

two-thirds as active as slices. The supernatant solution had a relatively low activity. Repeated washing of the particles with standard medium resulted in a partial loss of activity. The third column in Table II indicates the degree of reproducibility with replicate experiments.

Effect of Various Preliminary Treatments (Table III)—The importance

of tonicity is shown by the fact that homogenizing in the presence of distilled water destroyed most of the activity. The need for structural integrity is further indicated by the markedly lowered rate of C^{14} uptake following lyophilizing or repeated freezing and thawing. Similar results were obtained in connection with PAH synthesis (6) and were interpreted in terms of changes in the state of aggregation of nucleoprotein.

TABLE IV

Influence of Various Substances on Uptake of C^{14} by Insoluble Particles of Liver Homogenate

The particles were washed three times with saline-bicarbonate solution.

Change in composition of standard medium	Relative radio-activity of protein
None (standard medium)	100†
Medium replaced by saline-bicarbonate solution	35
Potassium omitted from standard medium	80
Phosphate " . . .	87
Magnesium " . . .	85
Calcium omitted . .	43
Glucose " . .	79
Citrate " . .	77
Both glucose and citrate omitted.	78
Citrate replaced by 0.002 M fumarate	87
5×10^{-6} M cytochrome C added to medium	88
5×10^{-4} M cozymase added .	88
0.002 M pyridoxal added	98
O ₂ replaced by N ₂ atmosphere	10
0.001 M ATP added; N ₂ atmosphere	15
0.002 M L-phosphoglyceric acid added; N ₂ atmosphere	15
Cytochrome + ATP + fumarate + cozymase added..	67
0.005 M cyanide added.	5
0.005 M azide added . .	5
0.1% amino acid mixture added . .	65

† This activity averaged 17.1 counts per minute per mg. of protein per hour of reaction time with homogenates prepared on different days.

The homogenate lost activity slowly at refrigerator temperatures and retained only 18 per cent of its original activity after 24 hours at 3°.

Since intact blood cells were present in the homogenates, the possibility existed that these cells were responsible for the C^{14} uptake. However, the activity was not lowered when *perfused* liver was used in the preparation of the homogenate. In addition, it was found that neither rat (non-nucleated) nor chicken (nucleated) erythrocytes had significant activity when incubated with glycine* in the standard nutrient solution.

Importance of Various Metabolites (Table IV)—The particles, freed of

soluble constituents by three washings with saline-bicarbonate solution, provided a favorable material for a study of the rôle of these same constituents in the process of C^{14} incorporation into the homogenate protein. When incubated in saline-bicarbonate solution, the particles had only a third as much activity as when tested in the presence of the standard medium.

Of the individual inorganic ions, the omission of calcium resulted in the greatest decrease in the rate of C^{14} uptake. In contrast to this observation, the omission of calcium led to a *stimulation* of PAH synthesis (9).

The omission of either glucose or citrate (or both) from the medium resulted in a decrease of approximately 20 per cent in the rate of C^{14} incorporation. Fumarate appeared to substitute only partially for citrate in the presence of glucose. In the case of PAH synthesis, fumarate was the most effective of a number of metabolites (chiefly components of the tricarboxylic acid cycle) in stimulating the reaction (9).

Other indications that the mechanism of C^{14} incorporation differs from that of PAH synthesis are findings that neither cytochrome *c*, under aerobic conditions, nor adenosine triphosphate (ATP), anaerobically, stimulated the former process significantly. Likewise, it was found that ATP failed to reactivate preparations which had previously stood for 24 hours in the refrigerator. Phosphoglyceric acid, which can act as an ATP generator in certain cases (10), did not promote C^{14} uptake anaerobically. Cohen and McGilvery (6) regard ATP as essential to PAH synthesis.

Pyridoxal, concerned with phosphate transfer in connection with decarboxylase and transaminase activity, had no effect on C^{14} utilization by homogenate protein, and had a slight depressant action at higher concentrations.

A combination of cytochrome, cozymase, ATP, and fumarate exerted a *depressant* effect on C^{14} utilization, whereas a like combination stimulated PAH synthesis.

The results obtained thus far permit no definite conclusions concerning the mechanism of the glycine* utilization process. There is as yet no evidence that phosphate bond energy is required. However, some type of oxidative mechanism is certainly suggested by the fact that oxygen is required and that cyanide and azide are strong inhibitors of the reaction.

It is worthy of note that the addition of a mixture of all of the various amino acids (except glycine) depressed, rather than stimulated, the rate of C^{14} uptake. In this connection, it may be noted that C^{14} incorporation occurs with washed particles, presumably freed from amino acids. However, certain of the acids other than glycine can apparently be derived from glycine (5), while autolysis may supply others. The C^{14} of the homogenate protein is distributed among several amino acids (5). Nevertheless, it cannot be concluded that the incorporation of a given amino acid requires the participation of other amino acids.

SUMMARY

The incorporation of C^{14} into the protein of cell-free homogenates of rat liver has been observed, following incubation with labeled glycine at 37° in an oxygen atmosphere. The process appears to be enzymic in nature. It is inhibited by heat, cyanide, azide, and anaerobic conditions. The rate of incorporation of C^{14} is dependent upon the glycine concentration, time of reaction, and pH of the medium.

The enzyme system which promotes the utilization of the C^{14} of glycine is associated with the insoluble particles of the homogenate. The process is promoted by certain inorganic ions: magnesium, phosphate, potassium, and particularly calcium. Of a number of organic substances tested, glucose and citrate exerted a slight stimulatory effect. Adenosine triphosphate, cytochrome, pyridoxal, and cozymase, on the contrary, caused a slight inhibition.

Addendum—Very recently Mr. E. Peterson of our laboratory has obtained a several fold increase in the rate of C^{14} incorporation by reducing the volume of the incubation mixture to 0.33 ml. without altering the amount of homogenized liver or liver particles customarily employed. Concentrated particles are about 3 times as active as liver slices, and since smaller amounts of glycine* (0.03 mg.) are employed, the efficiency is such that approximately 10 per cent of the C^{14} is incorporated within 1 hour.

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DISTRIBUTION OF RADIOACTIVE CARBON AMONG CERTAIN AMINO ACIDS OF LIVER HOMOGENATE PROTEIN, FOLLOWING UPTAKE EXPERIMENTS WITH LABELED GLYCINE*

BY THEODORE WINNICK, INGRID MORING-CLAESSON,[†] AND DAVID M. GREENBERG

(From the Division of Biochemistry, University of California Medical School, Berkeley)

(Received for publication, February 17, 1948).

Following the demonstration that C^{14} -labeled glycine (glycine*) is taken up by the protein of cell-free homogenates of rat liver (1), it was pertinent to demonstrate that the radioactivity of the protein was due to incorporated amino acids and to identify the latter.

Frantz, Loftfield, and Miller (2), in studying the uptake of alanine* by liver slices, showed that a large percentage of the radioactivity of the protein was due to alanine*. They added relatively large proportions of inert alanine carrier to hydrolysates of the protein, and then isolated and purified the alanine by repeated recrystallization.

A technique similar to that of Frantz and coworkers was employed by us to investigate the distribution of C^{14} in homogenate protein, following incubation experiments with glycine*. The results have indicated that a large portion of the C^{14} is contained in serine and that glycine itself accounts for only about one-eighth of the total radioactivity of the protein.

In order to determine with certainty the identity of the radioactive substance in the isolated glycine and serine, these amino acids were converted into suitable derivatives. Also, degradation procedures were used in certain cases to determine the position of the C^{14} in amino acid molecules.

EXPERIMENTAL

Labeled Glycine— $C^{14}H_2(NH_2)COOH$ containing 4.57 microcuries (560,000 counts per minute per mg. in the Geiger-Müller counter) was synthesized by Ostwald (3) from methyl-labeled acetate (4).¹

Homogenate Protein—0.5 gm. of liver homogenate protein was prepared by incubating insoluble cell particles with $C^{14}H_2(NH_2)COOH$ under suitable conditions (1).

* Aided by grants from the American Cancer Society (recommended by the Committee on Growth of the National Research Council), the National Cancer Institute, United States Public Health Service, and the Rockefeller Foundation.

[†] Permanent address, Upsala University, Upsala, Sweden.

¹ Dr. Rosemarie Ostwald in collaboration with Professor M. Calvin of the University Radiation Laboratory.

Isolation of Amino Acids—The protein was hydrolyzed with 15 parts of 6 N hydrochloric acid in a sealed tube in an autoclave for 18 hours at 17 pounds pressure. The hydrolysate was decolorized with carbon, evaporated to dryness, redissolved in water, and freed from residual chloride with silver carbonate. Separate aliquots, each representing 75 mg. of the original protein, were mixed with 300 mg. quantities of various pure amino acids that served as carriers. The different amino acids were then isolated by the following procedures: L-Glutamic and L-aspartic acids were precipitated from concentrated aqueous solutions at pH 3.2, while ethyl alcohol was added to promote the crystallization of glycine, DL-alanine, and DL-serine from neutral solutions. The amino acids were recrystallized (a specified number of times) to constant radioactivity. L-Arginine was first separated as the diflavinate and then converted to the monohydrochloride.

TABLE I

Derivatives of Glycine, Serine, and Alanine

The derivatives were prepared from four times recrystallized alanine and seven times recrystallized glycine and serine.

Compound	M.p		Nitrogen	
	Found	Reported†	Found	Theory
	°C.	°C.	per cent	per cent
Acetyl glycine	205–206	207–208 (5)	11.45	11.95
Phenylureidoserine	164–166	168–169 (6)	12.22	12.49
Benzoylalanine	161	160–161 (7)	7.09	7.22

† The figures in parentheses refer to the bibliography.

Nitrogen determinations on the purified amino acids gave results which agreed in all cases with theoretical values to within 0.5 to 1 per cent.

Purification of Glycine and Serine—50 to 100 mg. quantities of seven times recrystallized glycine and serine were converted to the acetyl (5) and phenylurea (6) derivatives, respectively. These derivatives were recrystallized twice from hot water and dried at 100°. Their purity was established by melting point and nitrogen determinations (Table I). Data are also given for benzoylalanine, prepared by the method of Dunn and coworkers (7). This compound was used in pilot separation experiments, but not in connection with protein analysis.

Degradation of Serine—The C¹⁴ of the carboxyl group was determined by treating 15 mg. of serine (recrystallized seven times) with ninhydrin, according to the procedure of Van Slyke, MacFadyen, and Hamilton (8), and collecting the evolved carbon dioxide as barium carbonate.

The C¹⁴ of the β -carbon was measured by oxidation of 15 mg. of the serine with periodate and isolation of the resulting formaldehyde as the dimedon derivative (9).

Degradation of Alanine—The C^{14} of the carboxyl group was measured as in the case of serine.

The C^{14} of the α - and β -carbon atoms was determined by treatment of 15 mg. of four times recrystallized alanine with ninhydrin and isolation of the resulting acetaldehyde as the 2,4-dinitrophenylhydrazone. The method of Van Slyke and coworkers (8) was used, except that 2 ml. of saturated dinitrophenylhydrazine in 2 N hydrochloric acid, instead of barium hydroxide, were placed in one arm of the apparatus. After the solution was cooled, the hydrazone was collected, washed with cold 2 N acid, and dried in air.

Radioactivity Measurements—The technique of collecting and counting samples is given in the preceding paper (1). Samples of serine which had relatively high activities gave counts of 3 to 4 times the background strength and could be measured readily with an error in counting of less than 3 per cent. The corresponding error was 5 to 7 per cent with other radioactive preparations. The reproducibility of measurements with various substances is indicated by the values recorded for successive stages of purification.

The following formula was used to calculate the percentage of the total radioactivity of the proteins due to each amino acid:

% total C^{14} due to amino acid A

$$= \frac{\text{Specific activity of } A \text{ (diluted with carrier)}}{\text{Specific activity of original protein}} \times \frac{\text{total weight of } A}{\text{weight of protein}} \times 100$$

The total weight of A was taken as 300 mg. (the quantity of A in the protein neglected), and the weight of protein as 75 mg. The term specific activity represents counts of C^{14} per minute per mg. of amino acid or protein.

This general procedure is similar to that outlined by Frantz and coworkers (2). It does not reveal the actual concentrations of C^{14} in the amino acids, unless the amino acid composition of the protein is also known.

RESULTS AND DISCUSSION

Table II indicates that less than 1 per cent of added glycine is retained by either serine or glutamic acid after five recrystallizations. Since aspartic acid resembles glutamic acid closely in properties and has a lower solubility in water, no difficulty would be anticipated in freeing it of glycine*. It is most improbable that glycine* would contaminate the isolated arginine.

Table III shows that only about 11 to 12 per cent of the total C^{14} in the protein of liver homogenate was due to glycine*.³

Approximately 60 per cent of the C^{14} was due to serine. This radioac-

³ This same conclusion was reached through separate chromatographic experiments in which hydrolysates of labeled homogenate proteins were eluted from charcoal columns.

TABLE II

Removal of Small Proportions of Glycine from Serine, Glutamic Acid, and Alanine*

3 mg. of glycine* (15,000 counts per mg. per minute) were added to 300 mg. of inactive amino acid. Glutamic acid was recrystallized repeatedly from hot water and washed each time with cold water. Alanine and serine were recrystallized from aqueous solution by the addition of alcohol and were washed with alcohol. In a separate experiment, a mixture of 3 mg. of glycine* and 300 mg. of alanine was benzoylated, and the isolated product recrystallized repeatedly from hot water. The results are expressed in counts per minute per mg.

Amino acid present in excess	Initial radio-activity due to glycine*	Activity after 4 crystallizations	Activity after 5 crystallizations
Serine	150	2.0	0.9
Glutamic acid ..	150	0.8	0.4
Alanine	150	70	
Benzoylalanine....	150	40	

TABLE III

Distribution of C¹⁴ among Certain Amino Acids of Liver Homogenate Protein†

Substance isolated		C ¹⁴ content of amino acid	Per cent of total C ¹⁴ of protein due to amino acid
		<i>counts per min. per mg.</i>	
Glycine	5th recrystallization	0.74	12.0
	6th "	0.70	11.4
	7th "	0.75	12.2
	Acetyl derivative	0.67‡	10.9
Serine	5th recrystallization	3.98	64.5
	6th "	3.75	61.0
	7th "	3.68	59.5
	Phenylurea derivative	3.58‡	58.0
Alanine	COOH group	0.00	0.0
	β -Carbon (formaldehyde)	0.40	6.5
	COOH group	0.00	0.0
	α - + β -carbons (acetaldehyde)	0.00	0.0
Glutamic acid	5th recrystallization	0.09	1.5
Aspartic acid	5th "	0.07	1.1
Arginine		0.05	0.8

† The activity of this material corresponded to 24.65 counts per minute per mg.

‡ This value was calculated from the observed C¹⁴ content of the derivative and its weight relative to that of the amino acid.

tivity was contained chiefly in the α -carbon position, since there was none in the carboxyl group and only a low concentration in the β position of the molecule. These results are consistent with the view that serine was

formed by a condensation of the methyl-labeled glycine (or a related 2-carbon derivative) with a second substance which provided the β -carbon. The process may represent a reversal of that described by Shemin (10), whereby the carboxyl and α -carbon atoms of serine give rise to glycine in rats and guinea pigs, with glyoxylic acid as a possible intermediate. Recently Ehrensward and associates (11) have observed the formation of serine from glycine in yeast.

Alanine isolated from the protein hydrolysate of the liver homogenate with carrier was observed to be radioactive.³ This activity was retained after benzylation. However, degradation experiments yielded no C^{14} in the α -, β -, or carboxyl carbon atoms. These findings, together with the

TABLE IV

Inhibitory Effect of Non-Radioactive Amino Acids on Utilization of Glycine by Liver Homogenate*

3 ml. samples of a preparation of washed, insoluble homogenate particles in standard medium (1) were incubated with 0.2 mg. of $C^{14}H_2(NH_2)COOH$ and 2.0 mg. of an inactive amino acid for 1 hour at 37°. The protein fraction was isolated as described in the preceding paper (1).

Inactive amino acid added	C^{14} content of homogenate protein <i>counts per min. per mg.</i>	Relative incorporation of C^{14} into protein*
None..	13.3	100
Glycine.	1.4	10
DL-Alanine.....	7.4	56
DL-Serine.. . . .	1.6	12
DL-Threonine	9.5	72

* The control experiment (no inactive amino acid) is expressed as 100.

observations in Table II, lead to the conclusion that the alanine was contaminated with C^{14} -containing amino acids.

The low C^{14} concentrations found in glutamic and aspartic acids and in arginine indicate that the fixation of carbon dioxide, derived from the decomposition of the glycine*, was responsible for only a minor fraction of the C^{14} incorporated into the liver homogenate protein. This result may be in part a consequence of the use of bicarbonate in the nutrient medium during the incubation process. The bicarbonate would have diluted the radioactive carbon dioxide greatly.

Table III accounts for approximately 75 per cent of the C^{14} of the protein. Losses probably occurred during hydrolysis. Also other amino acids, in addition to those isolated, may have contained C^{14} .

³ The radioactivity could not be removed by treatment with norit A.

In connection with the conversion of glycine to serine, it is noteworthy that the addition of excess inactive glycine or serine resulted in an inhibition of approximately 90 per cent in the degree of C^{14} incorporation into homogenate protein (Table IV).⁴ This effect can be ascribed to a dilution of radioactive by inactive amino acid. Alanine and threonine, which are probably not derived from glycine, inhibited C^{14} uptake to a much lesser degree.

Lastly, it may be mentioned that results in accord with those in this paper were obtained when carboxyl-labeled glycine (3) was used as a source of C^{14} in homogenate protein. In this case, almost 100 per cent of the C^{14} could be accounted for as carboxyl groups of the hydrolyzed protein. Serine again accounted for more than half of the C^{14} .

SUMMARY

Radioactive protein, derived from incubation of liver homogenate with C^{14} -labeled glycine, was hydrolyzed and subjected to analysis. Amino acid isolation experiments with non-radioactive carriers indicated that only 11 to 12 per cent of the C^{14} in the protein was due to glycine itself. The major portion, approximately 60 per cent, of the isotopic carbon was found in serine, derived from the glycine. Low concentrations of C^{14} were found in glutamic and aspartic acids and in arginine. Alanine contained no C^{14} .

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⁴ Indication that free serine was formed in the homogenate system was obtained by adding inactive serine carrier to a trichloroacetic acid filtrate of the reaction mixture. After nine recrystallizations, the radioactivity of the carrier accounted for 8 per cent of the total C^{14} originally employed, whereas in the parallel experiment with inactivated homogenate (zero reaction time), the serine carrier had virtually zero radioactivity (less than 0.3 per cent of the total C^{14}).

THE INFLUENCE OF pH, DYE, AND SALT CONCENTRATION ON THE DYE BINDING OF MODIFIED AND UNMODIFIED FIBRIN*

BY MARCUS SINGER AND PETER R. MORRISON†

(From the Departments of Anatomy and Physical Chemistry, Harvard Medical School,
Boston)

(Received for publication, January 23, 1948)

The combination of cationic and anionic dyes with proteins of tissues is difficult to interpret chemically because the reactions depend upon many factors. Among these are the physicochemical conditions of staining and the chemical structure, purity, and physical state of the protein.¹ The study of these factors may be simplified by employing "model tissues" but the solid proteins heretofore available for such study (6-9) have certain inherent disadvantages (*e.g.*, wool is a highly specialized fibrous protein and gelatin a degraded one). However, fibrin, a solid protein recently made available in the form of a film (10, 11), is better suited for this purpose. Although it is in a solid and insoluble state, fibrin film must be considered a native undenatured protein, since the fibrinogen from which it is prepared is completely soluble and the conversion of fibrinogen into fibrin by homologous thrombin is chemically identical with the physiological formation of fibrin within the body. Homogeneous sheets of film may be prepared at uniform and desirable thicknesses, and these sheets may be stained under suitable experimental conditions. The amount of dye combined with the fibrin may be measured photometrically. In the present report, the interactions of fibrin with cationic and anionic dyes and the effects thereupon of variations of concentration of dye, of ionic strength, and of pH of the staining solution will be considered. The effects of three denaturing treatments upon the subsequent interaction of fibrin and dye will also be described.²

* This paper is No. 71 in the series "Studies on plasma protein" from Harvard Medical School, on products developed by the Department of Physical Chemistry, from blood collected by the American Red Cross. The investigation was aided by a grant to the Department of Anatomy from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

† Present address, University of Wisconsin, Madison, Wisconsin.

¹ A preliminary report of the present study was made at the annual meeting of the American Association of Anatomists (1) and some initial applications of this study to tissue sections are reported elsewhere (2-5).

² The effects of heat treatments on fibrin film have been reported elsewhere (12); it was observed that the affinity for dye under a single fixed set of conditions is a very sensitive criterion of modification of the protein.

Material and Methods

Fibrin Film—Fibrin film³ was prepared from human fibrinogen and thrombin by methods described elsewhere (10, 11). Films of different thicknesses have been used; with films subjected to the same treatments the thickness varies with the weight of fibrin per unit area (mg. per sq. cm.). However, the equilibrium swelling of the film is changed by various modifying treatments. Consequently, the uptake of dye has been studied in its relation to both the thickness of the film and the concentration of the fibrin. Most of the films used contain about 3 mg. of fibrin per sq. cm., although some ranged as high as 16 and as low as 1 mg. per sq. cm.

In addition to the native material, modified fibrin was also employed. Before being subjected to one of three modifying treatments, fibrin film was dried on a ferrotype plate to a moisture content of 10 to 20 per cent to give a perfectly smooth and flexible but inelastic sheet.⁴ One of these treatments involved exposure to steam for 1 minute at 100°, another for 20 minutes at 120° (15 pounds per sq. in.). Upon heating, the film becomes more dense and rigid, a phenomenon which has been attributed to the formation of cross-links in the fibrin network (12). The third treatment involved immersion in 10 per cent formaldehyde for 16 hours at room temperature, which produced an even more strongly cross-linked material with a higher water-equilibrated fibrin content and even less elasticity. Comparison of the properties of the unmodified starting material with those of the modified film allowed the changes in dye uptake to be defined and related to the concomitant changes in the physical and chemical characteristics of the protein.

Unmodified fibrin film swells markedly in acid or alkaline solutions. This swelling is greatly increased in the presence of dyes, so that at even moderate acidities the film may become jelly-like or dissolve entirely. For this reason it was not possible to study the unmodified film over a wide pH range. The modified films, strongly cross-linked by the treatment, swelled little or not at all (12).

The apparent isoelectric point of fibrin was determined by measuring the rate of electrophoretic migration in buffers. The electrophoresis cell described by Abramson *et al.* (13), with copper-copper sulfate electrodes, was employed. Samples of dried film were ground in a mortar and aliquots were suspended in large volumes of dilute acetate or phosphate buffers (ionic strength, 0.02); the suspension was concentrated by centrifugation before being placed in the apparatus.

³ In this paper the term *film* when used alone always implies fibrin in the form of a film.

⁴ If the film is not dried before modification, the resulting product is opaque and weak.

Buffers—Since dye binding was studied between pH 2 and 10, it was necessary to use several buffer systems. A borate system (14) was employed above pH 8, phosphate buffers (15) between pH 8 and 5.5, an acetate system (15) between pH 5.5 and 3.8, and hydrochloric acid-sodium chloride mixtures below pH 3.8. The use of these various systems complicates the interpretation, since the dye-protein interaction may be influenced by the ionic strength, by the concentration of positive and negative charges, and by specific ion effects. When the influence of phosphate and acetate buffers was compared at a single pH and ionic strength, there was excellent correspondence in the uptake of dye (Table I). This apparently indicates that Donnan and specific ion effects are of little importance. However, at this pH, which is near the isoelectric point of fibrin, Donnan and specific ion effects may be less than at a pH where the net charge on the protein is

TABLE I

Effect of Buffer Composition on Uptake of Dye by Fibrin Film

At pH 5.50; ionic strength, 0.04; dye concentration, 2.5×10^{-4} M.

The amount of bound dye is expressed as optical density per mg. of fibrin per sq. cm.

Treatment of fibrin	Amount of methylene blue bound to fibrin				Amount of orange G bound to fibrin			
	Acetate solution	Phosphate solution	Chloride solution	Citrate solution	Acetate solution	Phosphate solution	Chloride solution	Citrate solution
Unmodified	0.07	0.15	0.10	0.17	0.58	0.45	0.49	0.43
1 min. at 100°	0.25	0.25	0.24	0.41	1.27	1.14	0.87	1.14
20 min. at 120°	0.35	0.33	0.38	0.59	1.29	1.33	1.13	1.01
Formaldehyde	2.21	2.00	0.73	0.80	0.41	0.40	0.19	0.29

higher. Yet, the curves relating dye binding to pH exhibited no discontinuities at the point corresponding to transitions from one buffer system to another (Figs. 3 to 6). Nevertheless, Donnan and specific ion effects cannot be entirely neglected, since considerable discrepancies were obtained when the effects of citrate and chloride systems were compared (Table I).

Dyes—An acid dye, orange G, and a basic dye, methylene blue, were used. The dye samples used were certified by the Biological Stain Commission. Methylene blue, tetramethyldiaminodiphenazthionium or tetramethylthionine, was obtained as the chloride salt, $C_{16}H_{18}N_3SCl$, in a purity of 84 per cent. Orange G, $C_{16}H_{10}N_2O_7S_2Na_2$, is the disodium salt of a strongly acid dye, benzene-azo-2-naphthol-6,8-disulfonic acid; the purity of the sample was 89 per cent.

The dye concentrations covered a fairly wide range (1 to 125×10^{-5} M) the limits of which were set by the technical difficulties of measuring the

bound dye. These limits were extended somewhat by the use of films of different thickness. The concentration of the dye solution in equilibrium with the stained film was measured with a Klett photoelectric colorimeter at the termination of each experiment. Since large volumes of dye solution were used in staining the film, the final dye concentration never deviated from the initial by more than a few per cent.

Staining Procedure—Pieces of film of the four types, approximately 2 cm. square and cut to various shapes for identification, were suspended in 1500 cc. of dye solution at a temperature of $25^{\circ} \pm 0.1^{\circ}$. The solution was stirred continuously, and at 24 hour intervals the films were removed and their dye content measured. If the optical density of the film had increased no more than 5 per cent since the previous reading, this reading was taken as the equilibrium value. With the thinner films equilibrium was invariably reached within the first 48 hours.

The optical density of the films was determined with a Pulfrich photometer equipped with a green filter (Zeiss L-II). Before measurement, each film was mounted with slight pressure in a drop of buffer solution between two glass slides. To decrease cloudiness, the unmodified films were briefly immersed in glycerol. After such treatment, unstained film transmitted 90 to 95 per cent of the incident light. In practice, with this unstained control film in place, the light was adjusted until a meter reading of 100 was obtained. The percentage absorption of the stained films was then read directly and the optical density calculated as the negative logarithm of the transmittance.

Disks of about 1.5 sq. cm. in area were punched from the stained films and, after the glycerol was washed from the unmodified films, the films were dried at 110° for 16 to 48 hours and then weighed. The dry weight was taken as fibrin and the opacity was then expressed as optical density per mg. of fibrin per sq. cm. In these experiments the weight contribution of the bound dye was always negligible.

Results and Comments

Relation of Dye Uptake to Amount of Fibrin and to Film Thickness—The relations between the amount of dye bound, the film thickness, and the film weight were studied under several conditions in a series of films ranging from 2 to 25 mg. per sq. cm. Provided equilibrium had been attained, the amount of methylene blue bound to films of various thicknesses was proportional to the amount of fibrin (Fig. 1). These results indicated that the dye was distributed uniformly throughout the film irrespective of thickness and was not in higher or in lower concentration near the surface.

Optical Density of Bound Dye—To determine the amount of dye corresponding to a particular optical density, the light absorption of known quan-

ties of fibrin-bound dye was measured. Pieces of fibrin film of known size and weight were immersed in different volumes of dye solution and the partly filled containers were rotated for 24 hours; the films were then removed and their optical densities determined. Finally, the concentration of the residual dye in the solution was measured and the amount of dye taken up by the film calculated. The quantities of fibrin and dye were so chosen that a considerable fraction of the dye was taken up from the solution.

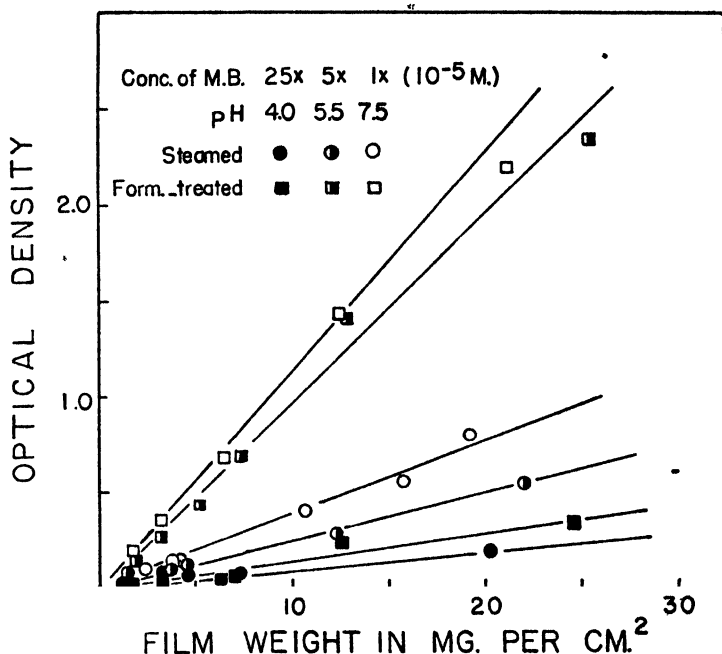


FIG. 1. The uptake of dye as a function of film weight. Equilibrated for 68 to 74 hours with two changes of buffer. The films represented by the upper two points may not have reached equilibrium. As noted in the figure, three molar concentrations of methylene blue were used: 25×10^{-5} , 5×10^{-5} , and 1×10^{-5} .

A protocol of one such experiment with methylene blue is presented in Table II. The results of all experiments with this dye showed that the optical density per mm per sq. cm. describes a single straight line corresponding to an absorption constant of 3.92×10^3 (Fig. 2; Table III). Therefore, the nature of the dye binding is independent of the degree of saturation, and consequently the interaction of methylene blue and fibrin adheres to Beer's law. The nature of the binding also appears to be independent of the treatment to which the film was exposed, since the various points (Fig. 2) define a single function, although different films and different staining

conditions were employed. Accordingly, the amount of bound dye will be calculated with this absorption constant.

The broken curve in Fig. 2 shows the optical densities of similar amounts of free dye. The small but definite difference between the two is of the same order as that observed by Michaelis and Granick (16) for the binding of methylene blue to nucleic acid. Similar displacements of absorption curves have been observed on binding other dyes to soluble proteins (17).

TABLE II

Protocol of One Experiment to Determine Absorption of Bound Methylene Blue
pH, 7.08; formaldehyde-treated fibrin film; concentration of dye, 9.8×10^{-5} mole per liter.

Volume of dye solution	Weight of fibrin	Final dye concentration	Dye bound	Dye bound per mg. fibrin	Optical density of film	Film weight	Optical density per mg per sq. cm.	Optical density per mm per sq. cm. $\times 10^{-2}$
cc.	mg.	mm per cc. $\times 10^3$	mm $\times 10^3$	mm $\times 10^5$		mg fibrin per sq. cm.		
10	268	0.51	0.93	0.35	0.20	15.4	0.013	3.72
20	249	0.73	1.82	0.73	0.41	15.7	0.026	3.56
40	236	0.95	3.52	1.50	0.86	15.1	0.057	3.80
80	219	1.57	6.56	3.00	1.44	13.8	0.104	3.49
160	280	1.87	12.70	4.54	2.70	15.6	0.173	3.81

TABLE III

Light Absorption of Methylene Blue and Orange G When Bound to Fibrin under Several Conditions

Treatment of fibrin	Orange G		Methylene blue	
	pH	Optical density per mm per sq. cm. $\times 10^{-2}$	pH	Optical density per mm per sq. cm. $\times 10^{-2}$
Formaldehyde	4.0	2.77	7.0	3.69
Heated	4.0	2.35	7.0	3.98
	6.5	3.87	10.0	3.77

In terms of the amount of absorbing substance, these values are equivalent to the absorption of a molar solution of dye at a depth of 1 cm.

Changes in the absorption spectrum of orange G were observed when the dye was made more concentrated in free solution and when it became bound to the protein. And, unlike those of methylene blue, the absorption constants of bound (Table III) and free orange G varied according to the pH and, in the case of the bound dye, also according to the film used.⁵ Conse-

⁵ Less variation was observed when a blue filter was used (Zeiss L-III) which transmits wave-lengths closer to the absorption maximum. However, at these wave-lengths the eye is less sensitive than at those used here.

quently, for exact conversion of the optical density into moles of dye it would be necessary to correct for the chromatic changes under each set of staining conditions. An average absorption constant of 3.0×10^4 was used here.

Influence of pH—The effect of pH on dye binding is shown in Figs. 3 and 4. The acid dye, orange G, is bound strongly at low pH and, as the pH is raised, dye binding falls off until no staining can be observed. This fall

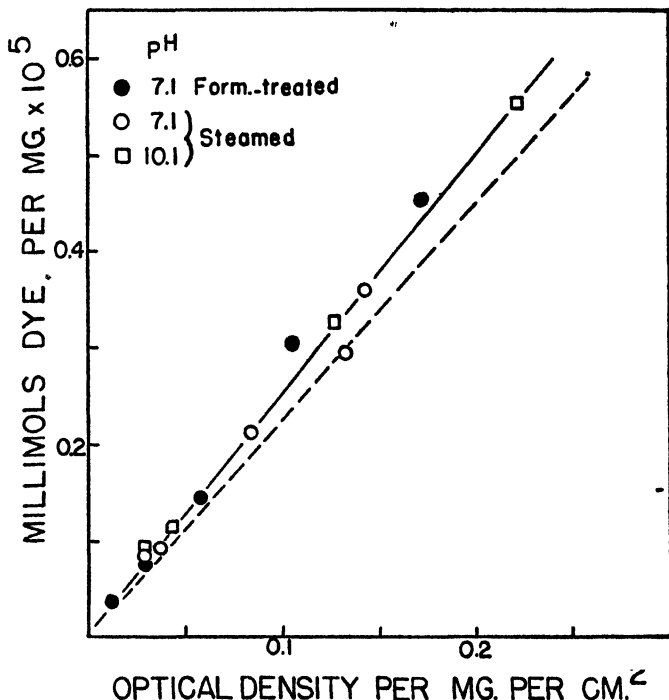


FIG. 2. The relation between optical density and millimoles of methylene blue bound to the fibrin film. The broken curve represents the optical densities of similar amounts of free dye.

accompanies the progressive decrease in the net positive charge on the protein, but some acid dye is bound even with a net negative charge. The curves for the basic dye, methylene blue, are the reverse of those for orange G. Except for the formaldehyde-treated film, the change in binding of methylene blue with pH is more gradual than is that of orange G. This may be related to the more gradual slope of the titration curve of fibrin above pH 5.5.

The quantitative interpretation of the effects of pH, as well as of salt and dye concentration, will not be attempted here. The binding of dye by a

dissolved protein is evidently complex (17), and with solid proteins analysis is still more difficult, since the concentrations of dye and salt and the pH of

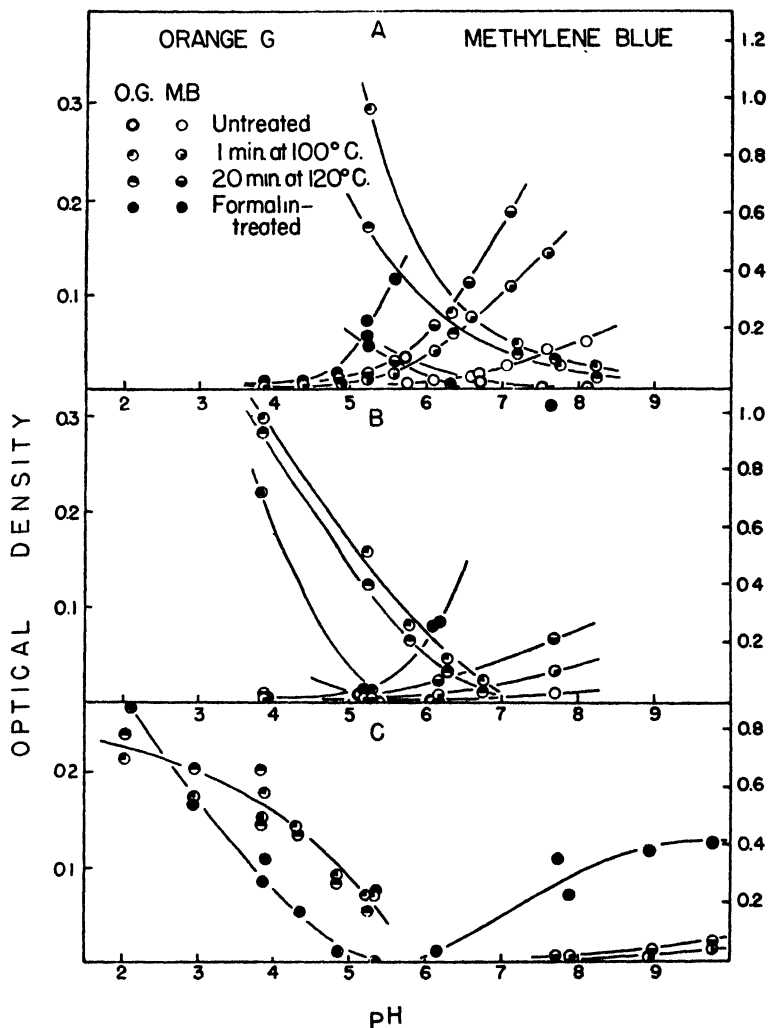


FIG. 3. The binding of methylene blue and orange G by various films at several dye concentrations. *A*, 2.5×10^{-4} M; *B*, 5×10^{-5} M; *C*, 1×10^{-5} M. Ionic strength, 0.04. The ordinate scales are so adjusted that a given point represents the same molar quantity of either dye. The factors for conversion have been taken from Table III. In the case of orange G the specific absorption showed variation with pH, dye concentration, and type of film; an average value was used.

the liquid phase actually in equilibrium with the protein are possibly very different from those of the staining solution. These relations have pre-

viously been described in terms of simple Donnan effects (8, 9) or by more involved treatments in which the "fiber water" is not considered an ordinary aqueous environment (18-20).

Recent studies on proteins have shown maximum binding for acid and basic dyes at pH 2 and 11, respectively (21), and the amount of dye bound was correlated with the number of dissociated groups on the protein. According to titration data and amino acid analyses, the maximum binding of methylene blue and orange G to fibrin should be respectively 192 and 122×10^{-5} mole per gm. (12, 22). Actually, however, the maximum observed values were less than 10 per cent of these figures; so that the amount of dye bound did not indicate the number of dissociated acidic and basic groups. Furthermore, at high or low pH the binding of dye varied with the dye concentration and the ionic strength of the solution (Figs. 3 and 4). Thus, the binding of orange G at pH 2 and ionic strength 0.04 (Fig. 4, B) is 8 to 9×10^{-5} mole per gm. for formaldehyde and heated films. At ionic strength 0.15 (Fig. 4, C) the dye uptake is approximately halved. Similar variations may be seen in the other figures, although in many instances the information is incomplete at very high or low pH and judgment must be limited to the trend of the curves.

Dye Concentration—The influence of dye concentration on binding to the four films is shown in Fig. 3 and to a single film in Fig. 5. No binding is seen in the unmodified film at the lowest dye concentration. There is a progressive decrease in staining of each film with decrease in dye concentration but these changes in dye binding do not follow the Langmuir adsorption isotherm. It is of interest that the two heat-treated films become indistinguishable at low dye concentrations.

Ionic Strength—The influence of ionic strength is shown in Fig. 4 for the four films and in Fig. 6 for a single film. In each instance less acid or basic dye was bound at the higher salt concentration. This may reflect merely a decrease in the concentration of free dye within or between the fibrillae of the film as a result of increased salt concentration. On the other hand, under conditions of low ionic strength and high charge on the protein the concentration of dye ion within these spaces might be increased many times over the outside concentration. Another possibility is that competition of the salt with the dye ions for binding sites on the protein would reduce the dye uptake. A steepening of the curves with decreased ionic strength (Figs. 4 and 6) is also observed in the titration of soluble and fibrous proteins (18).

Effect of Modification—The four films were compared under each set of experimental conditions. With one exception, modified fibrin was characterized by a greater affinity for either dye than was unmodified fibrin (Figs. 3 and 4). The one exception was formaldehyde-treated fibrin stained in a concentrated solution of orange G (Fig. 3). Furthermore, the

three modified films showed different affinities for the dye and the order of affinity was independent of the conditions of staining. Formaldehyde-treated film showed the greatest affinity for basic dye, followed in order by

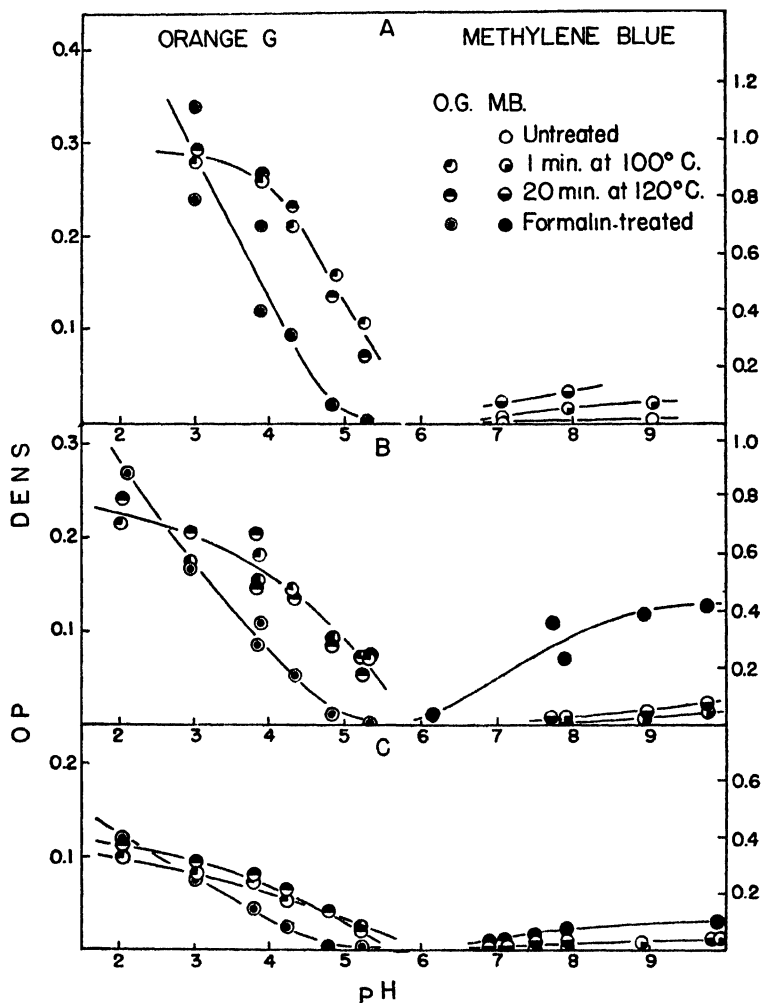


FIG. 4. The dye binding of various films at different ionic strengths. A, 0.01; B, 0.04; C, 0.15. Dye concentration, 1×10^{-5} M. Ordinate scales adjusted as in Fig. 3.

the strongly and mildly heated films. This succession was reversed for the acid dye. Under some conditions it was not possible to differentiate between the two heated films (Fig. 3, C; Fig. 4).

The alteration of the apparent isoelectric point of fibrin (Table IV) is of

interest in interpreting the effects of modification. The order of displacement of the isoelectric point caused by the various modifying treatments corresponds to a similar order of affinities for dye of the different modified

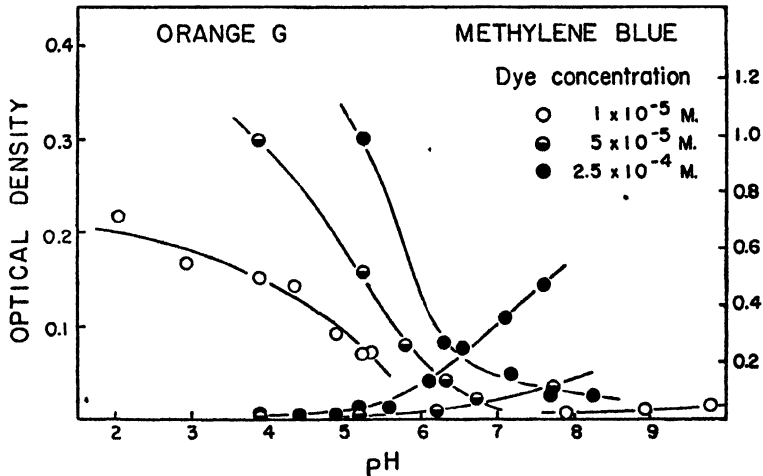


FIG. 5. The influence of three levels of dye concentration on the binding of dye by a single film, heated for 1 minute at 100°. Ionic strength, 0.04. Ordinate scales adjusted as in Fig. 3.

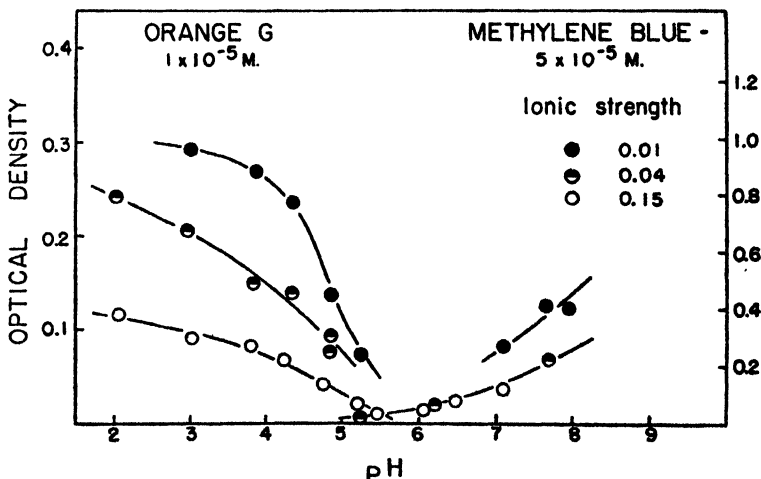


FIG. 6. The influence of ionic strength on dye binding in a single film, heated for 20 minutes at 120°. Ordinate scales adjusted as in Fig. 3.

films (compare Fig. 3 and Table IV). The greatest decrease in isoelectric point of the modified fibrin occurred in formaldehyde-treated film. This film also showed the greatest affinity for basic dye and the least for acid dye.

The observed effects of modifying treatments upon staining may be accounted for by two general mechanisms: first, a change in the physical structure or form of the protein, such that existing binding sites are made more available to dye (23, 24); and second, a change in the net charge resulting from either an increase or a decrease in the number of positively or negatively charged groups (*i.e.*, by a shift in the isoelectric point). In the former instance both dyes would be bound more readily, while in the latter one would expect an increase in affinity for one dye and a decrease in affinity for the other. Since the affinity of fibrin for both acid and basic dye was greatly enhanced by heat treatment, heat treatment must largely affect the availability of the dye binding sites. This change is presumably due to some physical reorganization in the fine structure of the fibrin, or perhaps of the molecular units themselves (*cf.* (23, 24)). In accord with this concept of a major effect from a physical reorganization, the dye binding curves of the mildly and strongly heated films are close or the same (Figs. 4 and 5),

TABLE IV
Isoelectric Point of Fibrin Films As Indicated by Electrophoretic Migration

Treatment of fibrin	Isoelectric point	Change in isoelectric point on treatment
Unmodified	6.0	
1 min. at 100°	5.7	0.3
20 min. at 120°	5.5	0.5
Formaldehyde	5.2	0.8

Ionic strength, 0.02.

although the degree of heating differed considerably. The small but definite difference between the curves of the two heated films evident under most conditions of staining suggests a slow progressive chemical modification as well, an effect also noted in previous studies (12). The progressive increase in basic and decrease in acid dye binding which appears with prolonged heating could result from a lowering of the isoelectric point; this possibility is supported by the electrophoretic studies.

Formaldehyde treatment rendered the film very basophilic (Figs. 4 and 5). This could result from the reaction of formaldehyde with amino groups to decrease the net positive charge (25). But in such a condition the affinity for acid dye should be reduced, while, actually, this affinity was approximately that of unmodified film. A possible explanation is that, in addition to the obvious combination with basic groups, formaldehyde may also increase the availability of all remaining charged groups by some physical modification of the structure similar to that described above for heat treatment.

SUMMARY

The affinities of films of human fibrin for the acid dye, orange G, and the basic dye, methylene blue, have been studied under controlled conditions of pH (2 to 10), ionic strength (0.01 to 0.15), and dye concentration (1 to 125×10^{-5} M). The extent of combination was a function of all these variables. Modification of the film by heat or formaldehyde treatment resulted in an increase in its affinity for acid and basic dye and a simultaneous or subsequent alteration in its relative affinities for the two dyes. The second effect was correlated with changes in the isoelectric point as measured by electrophoretic migration. The maximum binding of anionic and cationic dye at low and high pH, respectively, was much less than the estimated number of charged groups and this binding capacity varied with the ionic strength and concentration of dye.

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THE VITAMIN B₆ GROUP

XIII. AN IMPROVED PROCEDURE FOR DETERMINATION OF PYRIDOXAL WITH *LACTOBACILLUS CASEI**

By JESSE C. RABINOWITZ, NELL IRENE MONDY, AND ESMOND E. SNELL

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

(Received for publication, April 19, 1948)

Of all the known forms of vitamin B₆, pyridoxal, pyridoxamine, pyridoxine, pyridoxal phosphate, and pyridoxamine phosphate, only pyridoxal shows significant activity in promoting growth of *Lactobacillus casei* in a vitamin B₆-free medium (1, 2). For this reason, *Lactobacillus casei* was used to demonstrate the occurrence of pyridoxal in natural products, which also contain other forms of vitamin B₆ (3). It was pointed out, however, that the method used gave variable results from time to time; recorded assays were therefore the average of several individual determinations and were regarded as merely tentative.

Since then, additional information about the nutritive requirements of *Lactobacillus casei* and the liberation of vitamin B₆ from its bound forms has permitted development of a much more satisfactory method for determination of pyridoxal. This is described below.

EXPERIMENTAL

Basal Medium—The composition of the basal medium finally adopted is shown in Table I. This differs from the previous medium chiefly in addition of a tryptic digest of casein, in the omission of purine and pyrimidine bases, and in addition of higher levels of the other vitamins required by the test organism. Enzymatic digests of casein have been shown to contain substances which permit more rapid and extensive growth of *Lactobacillus casei* than is obtained in their absence (4). It is sometimes difficult, however, to free this material completely from vitamin B₆; hence it has not been used as the sole source of amino acids. Both the enzymatic and acid hydrolysates of casein were prepared by procedures previously described

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation. Some of the work reported here was carried out at the Biochemical Institute of the University of Texas. For Paper XII of this series, see Rabinowitz and Snell (1).

(4, 2). If, after the two charcoal treatments recommended in these procedures, the blank tubes still support some growth, the casein hydrolysates are stirred at pH 7.0 for 15 minutes with 10 per cent of Darco G-60 for a third time. However, this additional treatment has rarely been necessary. Adenine, guanine, and uracil, usually included in media of this type, have no effect on growth of *Lactobacillus casei* under the conditions used for assay and were omitted. If it is desired to use the medium for other lactic acid bacteria, many of which require one or more of these compounds under these conditions, they should be added at a level of 2.0 mg. each per 100 cc. of double strength medium.

TABLE I
Composition of Basal Medium

Substance	Amount per 100 cc. double strength medium (20 assay tubes)	Substance	Amount per 100 cc. double strength medium (20 assay tubes)
	mg.		mg.
Roberts and	1000	MgSO ₄ ·7H ₂ O	40
Snell (4))		NaCl	2
Acid-hydrolyzed casein	1000	FeSO ₄ ·7H ₂ O	2
Glycine	40	MnSO ₄ ·H ₂ O	2
Asparagine	20		7
DL-Tryptophan	20	Riboflavin	80
L-Cystine	40	Thiamine hydrochloride	40
Dextrose	2000	Calcium pantothenate	80
Sodium acetate*	1200	Niacin	80
K ₂ HPO ₄	100	p-Aminobenzoic acid	40
KH ₂ PO ₄	100	Folic acid	2
		Biotin	0.4

* The amount of sodium acetate indicated is in addition to that furnished by the tryptic digest of casein. When prepared as recommended (4), this latter ingredient supplies about 985 mg. of sodium acetate, which brings the total concentration of this substance to slightly over 2 per cent of the double strength medium.

The casein hydrolysates with added amino acids and the solution of the vitamins were kept in the refrigerator under toluene. These mixtures are also used for preparing the basal medium in the assay of pyridoxamine and pyridoxal with *Streptococcus faecalis* (5). The sugar and salts were kept in a solution frozen at -4°. These solutions may be stored for at least 2 months. The basal medium was prepared as required at twice its final concentration by mixing the calculated amounts of each of these solutions and adjusting the pH to 7.0 with potassium hydroxide.

Stock Culture and Inoculum—*Lactobacillus casei* was carried as stab cultures in yeast-dextrose agar (1 per cent yeast extract, 1 per cent glucose,

1.5 per cent agar) with incubation at 30°.¹ Stock cultures were transferred every 2 weeks from the yeast-dextrose agar to an enriched agar described by Hunter (6). After incubation in the enriched agar for 24 hours, the culture was transferred back to the yeast-dextrose agar. Cultures which were transferred less frequently often failed to grow satisfactorily in the inoculum tubes and the assay medium.

The basal medium supplemented with 100 mγ of pyridoxal hydrochloride per 10 cc. was used for growing inoculum. After 20 to 28 hours incubation at 37° the cells were centrifuged, resuspended in 10 cc. of 0.9 per cent sodium chloride solution, recentrifuged, and again suspended in 10 cc. of sterile saline.

Assay Procedure—Since pyridoxal is altered when autoclaved with amino acids (7), the usual procedure of adding the double strength medium to the samples and then autoclaving the sample and medium was not satisfactory. Instead, water sufficient to make a volume of 5 cc. after addition of the samples is autoclaved 10 minutes at 15 pounds pressure in 18 × 150 mm. Pyrex test-tubes which are covered with aluminum caps. After the tubes have cooled, the samples are added. Duplicate tubes containing 0, 1, 2, 3, 4, 5, 7, and 10 mγ of pyridoxal hydrochloride serve as the standard. In many assays the samples were sterilized separately, diluted appropriately in sterile water, and added aseptically. A great number of assays have been run without these precautions, and with no evidence of contamination, presumably because of the heavy inoculum and the short incubation period used.

In some assays, the possibility of contamination was avoided by diluting standard and samples appropriately with freshly autoclaved water, dispensing to the assay tubes, and reautoclaving these tubes containing the water and samples for 5 minutes.

The basal medium, previously autoclaved for 10 minutes at 15 pounds pressure and cooled, was inoculated with 1.0 cc. of the washed cell suspension per 100 cc. of the double strength basal medium. 5 cc. aliquots of this inoculated double strength medium were added aseptically to each tube. All tubes were then incubated at 37° for 22 hours, then steamed for 10 minutes to stop growth. After cooling, turbidities were determined with the Evelyn colorimeter adjusted so that the uninoculated basal medium read 100 per cent transmission with the 660 mμ filter. A typical standard curve is shown in Fig. 1.

Preparation of Samples—Although autoclaving the samples with the amino acids of the medium is readily avoided by the procedures described above, it is necessary to hydrolyze natural materials in order to make the

¹ The stock culture of *Lactobacillus casei* was incubated at 30° instead of the customary 37° simply for convenience. In a controlled experiment, no difference in the response of *Lactobacillus casei* to graded amounts of pyridoxal was observed whether the stock culture had been incubated at 30° or 37°.

vitamin B₆ available to the test microorganism (8), and the possibility existed that pyridoxal or its bound forms might give rise by transamination to pyridoxamine during this latter treatment.

Since glutamic acid has been shown to be the most active amino acid in this reaction (9), the effect of autoclaving pyridoxal and pyridoxal phosphate with glutamic acid under the conditions used for hydrolysis of the sample was determined. These results are shown in Table II. In confirmation of previous results (9), autoclaving pyridoxal with a relatively concentrated solution of glutamic acid at neutrality results in loss of activity for *Lactobacillus casei*, with only slight change in activity for *Streptococcus faecalis* or *Saccharomyces carlsbergensis*. These changes in

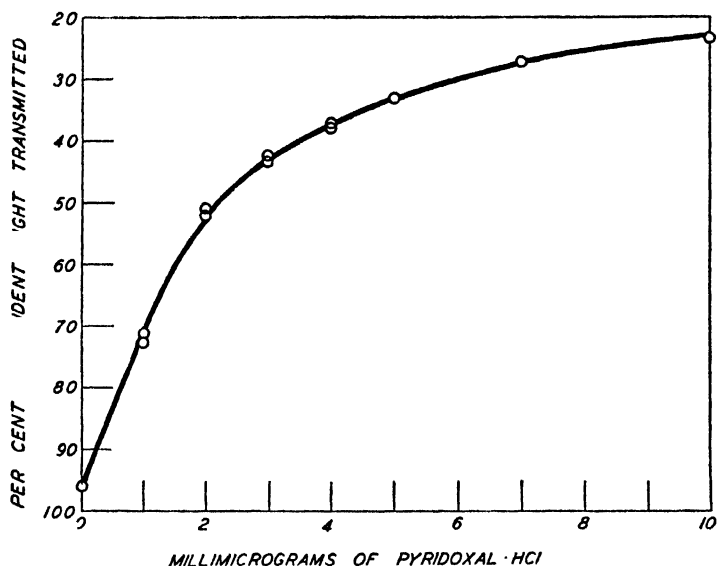


FIG. 1. Response of *Lactobacillus casei* to pyridoxal in the modified medium

activity result from the transformation of pyridoxal into pyridoxamine. The analogous transformation of pyridoxal phosphate to pyridoxamine phosphate also occurs under these same conditions. However, under the conditions recommended (8) for liberation of vitamin B₆ from natural products, *i.e.*, autoclaving in a volume of 180 cc. at pH 1.8, no loss in activity of pyridoxal or pyridoxal phosphate was observed when the same amount of glutamic acid was present; *i.e.*, transamination does not occur. The amount of glutamic acid used, 30 mg., gave a higher concentration of the amino acid than would normally be encountered, since only 50 mg. of a natural material are usually hydrolyzed in 180 cc. of acid. These data would indicate, then, that under these conditions the pyridoxal present in

the original sample is liberated by this hydrolytic procedure without significant transformation into pyridoxamine. Recovery experiments, cited below, provide additional evidence that this is the case.

TABLE II

Effect of Conditions on Transamination Reaction between Glutamic Acid and Pyridoxal or Pyridoxal Phosphate

Substance	Treatment*			Activity	
	Glutamic acid	Volume	pH	L. casei	S. faecalis†
	mg.	cc.		γ per γ	γ per γ
Pyridoxal HCl				1.0	1.0
Pyridoxamine 2HCl				<0.02	1.4
Pyridoxal HCl (2 γ)	30	2.0	7.0	<0.02	0.8
" " (2 ")	30	180	1.8	1.0	1.1
Pyridoxal phosphate (10 γ)	0	180	1.8	0.23	0.20
" " (10 ")†	30	2.0	7.0	<0.002	0.11
" " (10 ")	30	180	1.8	0.23	0.20

* The indicated amounts of pyridoxal or pyridoxal phosphate and glutamic acid were autoclaved at 20 pounds pressure for 5 hours in the volume of water and at the pH indicated.

† Determined by a method previously described (5).

‡ After treatment at pH 7.0, this sample was diluted to 180 cc., adjusted to pH 1.8, and autoclaved at 20 pounds pressure for 1 hour to hydrolyze the pyridoxal phosphate or pyridoxamine phosphate.

TABLE III

Recoveries of Pyridoxal Added to Natural Materials

Sample	Pyridoxal hydrochloride		Recovery
	Added	Found	
	γ per gm.	γ per gm.	per cent
Dried grass*	0	5.3	
" "	5.0	10.4	98
1:20 liver powder†	0	8.3	
1:20 " "	5.0	13.3	100
Dried yeast‡	0	7.2	
" "	5.0	12.7	110
Rat liver (fresh)	0	3.2	
" " "	5.0	7.9	94

* Cerograss.

† Wilson and Company product.

‡ Kindly supplied by Dr. L. Atkin.

Recovery Experiments—The results of recovery experiments in which known amounts of pyridoxal hydrochloride were added to representative natural materials are shown in Table III. In each case the control sample

and a duplicate sample supplemented with 5 γ of pyridoxal hydrochloride per gm. were autoclaved in 180 cc. of 0.055 N HCl at 20 pounds pressure for 5 hours (8), cooled, and neutralized with potassium hydroxide before assay. Recoveries of pyridoxal hydrochloride were within 10 per cent of the amount added in every case.

The pyridoxal content of several miscellaneous materials was determined by this procedure. The results are given in Table IV.

TABLE IV
Pyridoxal Content of Miscellaneous Natural Materials

Substance	Pyridoxal hydrochloride	Substance	Pyridoxal hydrochloride
	γ per gm. dry weight		γ per gm. dry weight
Chick liver	38	Liver concentrate*	16
" kidney	30	Yeast	8
" brain	11	Egg yolk	11
Rat liver	29	Whole wheat	2
" kidney	33	Carrot	2
" brain	9	Lettuce	12
Beef liver	7	Whole milk	γ per cc. 0.32

* Sharp and Dohme.

DISCUSSION

The inclusion of an enzymatic digest of casein in media used for vitamin assay with *Lactobacillus casei* has been shown to permit more luxuriant growth and greater acid production (4) than is obtained in media containing only acid hydrolysates of casein. "Strepogenin" (10) and perhaps other peptides (11, 12) are undoubtedly responsible for the added growth. However, when used in the medium for assay of pyridoxal, this modification may affect the specificity of the method, since it has been shown (11) that in the presence of such enzymatic digests D-alanine replaces pyridoxal for *L. casei*. The amount of D-alanine required to give half maximum growth of *L. casei* in the absence of pyridoxal is approximately 50 γ (11). L-Alanine is essentially inactive (11). Since the amount of pyridoxal hydrochloride required for half maximum growth is only about 0.002 γ , this effect of D-alanine would be significant only when the D-alanine content of natural materials was 10 to 25 thousand times the pyridoxal content; i.e., 1 to 5 per cent of the dry weight for most materials. In view of the limited distribution of D-amino acids in nature, the possibility of interfering effects from this source is negligible with most materials. Possibility of error from this source should, however, be kept in mind.

It was shown previously that the materials present in natural extracts which promote growth of *Lactobacillus casei* in vitamin B₆-free media corresponded in chemical properties to pyridoxal; *i.e.*, were destroyed by incubation with acetone and alkali, and with sodium cyanide and ammonium chloride. These properties are not shared by the other known forms of vitamin B₆, a fact which indicates the specificity of the assay method for pyridoxal. It would be expected, of course, that a variable proportion of the pyridoxal of natural materials might be present in loose, reversible combination with amino compounds, *e.g.*, Schiff's bases. Such compounds hydrolyze spontaneously under testing conditions to yield pyridoxal and show the activity for *Lactobacillus casei* to be expected from their pyridoxal content (13).

SUMMARY

An improved method for determination of pyridoxal with *Lactobacillus casei* is described. The principal change from the previous method is the inclusion in the medium of an enzymatic digest of casein which permits heavier and more rapid growth of the assay organism. While it has been shown that this modification permits the utilization of D-alanine in place of pyridoxal (11), the amounts of D-alanine required are not ordinarily encountered in the assay of natural materials.

Since pyridoxal and pyridoxal phosphate are easily transformed to pyridoxamine (7) or pyridoxamine phosphate (1) respectively, conditions are defined under which natural materials may be hydrolyzed to liberate pyridoxal without causing this transformation.

With this method of hydrolysis and the modified assay procedure, recovery experiments were carried out with a number of natural materials. Recoveries of pyridoxal were within 10 per cent of the amount added in all cases. The pyridoxal content of several tissues is recorded.

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D-GLUCOSE DEHYDROGENASE AND ITS CARRIER SYSTEMS*

BY B. EICHEL AND W. W. WAINIO

(From the Department of Physiology, New York University College of Dentistry,
New York)

(Received for publication, January 12, 1948)

In 1931 Harrison (1) tested the effect of D-glucose on the oxidation of hexose diphosphate with the intention of inhibiting the oxidation of the ester. He expected that due to similarities in structure the glucose might be adsorbed on the surface of the protein carrier and thus inhibit the reaction. However, instead of inhibition, he obtained a marked acceleration in the oxygen uptake. It appeared, therefore, that the liver preparation contained a dehydrogenase capable of oxidizing D-glucose. He was able to show that methylene blue could mediate the oxidation, and further that the cytochrome system could also serve as a carrier. Ogston and Green (2) were unable to verify Harrison's results with the cytochrome system. They showed instead that yeast flavoprotein could mediate the aerobic oxidation of the D-glucose, thus verifying the results of Adler and von Euler (3). Hawthorne and Harrison (4) strengthened their own position by demonstrating again that the cytochrome system of Keilin and Hartree (5) would catalyze the oxidation and by suggesting that Straub's flavoprotein was necessary for the full activity of the dehydrogenase-cytochrome system.

The experiments reported in this paper clarify the rôle of heart flavo-protein as a carrier with the liver dehydrogenase¹ system that oxidizes D-glucose. They further indicate that the cytochromes *c* and oxidase must be present to mediate the transfer of hydrogen from D-glucose dehydrogenase to atmospheric oxygen. The participation of the cytochromes *a* and *b* has not yet been verified.

EXPERIMENTAL

Preparations

D-Glucose dehydrogenase protein is contained in a preparation of partially purified lamb liver dehydrogenases, Preparation C, as previously reported by Wainio (6).

* Aided by a grant from the John and Mary R. Markle Foundation. With the assistance of S. Cooperstein, S. Kollen, and H. Hirschhorn.

¹ Dehydrogenase signifies D-glucose dehydrogenase protein and diphosphopyridine nucleotide.

DPN² was prepared from bakers' yeast³ by the method of Warburg and Christian (7), as reported by Klein (8).

Heart and liver flavoprotein were prepared from lamb tissue according to the method of Straub (9). However, instead of being ground in a mechanical mortar, the minced, washed tissue (the liver was not washed) was homogenized for 1 minute in a Waring blender with 0.02 M disodium hydrogen phosphate. The final flavoprotein solution was lyophilized from the frozen state to yield a stable preparation which was stored at 4°. The yield was approximately 57 mg. per kilo of minced heart and 38 mg. per kilo of liver.

*Catalase*⁴ (10) was used as the crystalline protein suspended in a solution of (NH₄)₂SO₄.

Cytochrome c was prepared according to Keilin and Hartree (11). The final preparation after dialysis was centrifuged for 5 minutes at 2500 × *g*. The supernatant was treated with 4 volumes of ice-cold acetone and centrifuged at 5° for 5 minutes (2000 × *g*). The precipitate was dried *in vacuo* to yield a stable protein.

Indophenol oxidase of Ogston and Green (2) was prepared from lamb heart muscle. The final acetate precipitate was suspended in an equal weight of 0.1 M phosphate buffer,⁵ pH 7.4.

Cytochrome oxidase was precipitated three times with acetate as reported by Hawthorne and Harrison (4). This preparation is a slight modification of the method of Keilin and Hartree (5). The details through the first acetate precipitate have been presented by Wainio, Cooperstein, Kollen, and Eichel (12). The first acetate precipitate from 50 gm. of heart muscle was suspended in 75 ml. of cold 0.04 M phosphate buffer, pH 7.0, and precipitated a second time with 12.5 ml. of cold 0.2 M acetate buffer, pH 4.6. After centrifugation in the cold for 10 minutes at 2500 × *g* the supernatant was discarded. The precipitate was resuspended in 50 ml. of the cold 0.04 M phosphate and precipitated with 8.3 ml. of cold 0.2 M acetate. After centrifugation for 10 minutes at 2500 × *g*, the supernatant was discarded and the precipitate suspended in an equal weight of 0.1 M phosphate buffer, pH 7.4. The suspension was filtered through four layers of cheese-cloth and stored at 4°.

Methylene Blue System

It was first decided to establish optimal concentrations of each enzyme constituent with the artificial methylene blue system, since Wainio (6) had

² DPN, diphosphopyridine nucleotide.

³ Fleischmann's.

⁴ Kindly supplied by Dr. J. B. Sumner.

⁵ Phosphate buffer in all instances refers to KH₂PO₄-Na₂HPO₄.

earlier demonstrated marked activity with the same liver protein preparation when DPN, triphosphopyridine nucleotide, and methylene blue were added. It has since been shown that DPN is the active coenzyme with the D-glucose dehydrogenase protein. A 10 mg. sample of the liver protein preparation dissolved in phosphate buffer was selected as the constant factor, and the concentrations of the other constituents that would make the dehydrogenase protein limiting were determined.

It was noted very early that 0.5 ml. of a 1:1000 solution of methylene blue in water was insufficient to support a maximal activity when DPN and D-glucose were at or above the optimal concentration. Decolorization occurred frequently and, therefore, 0.5 ml. of a 1:500 solution (1 mg.) was selected as being a sufficient amount of methylene blue.

TABLE I

Omission of Factors in Methylene Blue System and Effect of Flavoprotein

Body, 10 mg. of liver protein in 2 ml. of 0.1 M phosphate buffer, pH 7.4; 0.5 ml. of 1:500 methylene blue in water; 0.25 ml. of 0.1 M phosphate buffer, pH 7.4; flavoprotein, 1 mg. in 0.25 ml. of 0.1 M phosphate buffer, pH 7.4. Side arm, 0.25 ml. of 2 molal D-glucose in water; 1 mg. of DPN in 0.25 ml. of water. Center well, 0.25 ml. of 10 per cent NaOH. Gas phase, oxygen; temperature, 38°; rate of shaking, 100 per minute.

Vessel pair		1	2	3	4	5	6
		ml.	ml.	ml.	ml.	ml.	ml.
Body	Liver protein		2.0	2.0	2.0	2.0	2.0
	Methylene blue	0.5	0.5		0.5	0.5	0.5
	0.1 M phosphate buffer, pH 7.4	2.25	0.5	0.75	0.5	0.25	
Side arm	Flavoprotein						0.25
	D-Glucose	0.25	0.25	0.25		0.25	0.25
	DPN	0.25		0.25	0.25	0.25	0.25
		C.M.M.	C.M.M.	C.M.M.	C.M.M.	C.M.M.	C.M.M.
Oxygen up- take	60 min.	11	21	10	10	310	305
	120 min.	15	32	18	16	585	574

The effect of increasing the final D-glucose concentration from 0.01 molal to 0.231 molal was first studied. The substrate almost brought the activity to a maximum at a final concentration of 0.154 molal D-glucose. All subsequent experiments, therefore, were made at a 0.154 molal final concentration of D-glucose.

The effect of an increasing concentration of DPN on the activity of the system was tested at four concentrations. Essentially maximal activity was obtained at a DPN concentration of 1 mg. per vessel and all subsequent experiments, including those with flavoprotein, were run with DPN at this concentration.

Table I shows the effect of omitting the various factors of the methylene blue system, as well as the effect of added heart flavoprotein. The omis-

sion of the liver protein, DPN, methylene blue, or of D-glucose lowered the 120 minute oxygen uptake to between 15 and 32 c.mm. The system containing the dehydrogenase protein, DPN, D-glucose, and methylene blue consumed 585 c.mm. of oxygen in 120 minutes. The addition of 1 mg. of Straub's heart flavoprotein to this apparently complete, artificial system was without effect on the oxygen uptake. If flavoprotein does in fact catalyze this methylene blue system, it may be that the liver preparation already contains a sufficient amount of the flavoprotein, since it emits a greenish fluorescence in ultraviolet light.

Flavoprotein System

Adler and von Euler (3) in 1935 demonstrated that a crude flavin enzyme prepared from dry bottom yeast (Warburg and Christian (13)) could func-

TABLE II

Effect of Heart Flavoprotein Concentration

Body, heart flavoprotein in 0.25 ml. of 0.1 M phosphate buffer, pH 7.4; 0.5 ml. of water; 10 mg. of liver protein in 2 ml. of 0.1 M phosphate buffer, pH 7.4. Side arm, 0.25 ml. of 2 molal D-glucose in water, 1 mg. of DPN in 0.25 ml. of water. Center well, 0.25 ml. of 10 per cent NaOH. Gas phase, oxygen; temperature, 38°; rate of shaking, 100 per minute.

Heart flavoprotein per vessel	Oxygen uptake	
	60 min.	120 min.
mg.	c.mm.	c.mm.
8.0	290	535
4.0	240	440
2.0	160	300
1.0	110	200
0.5	80	140
0.25	50	90

tion as a carrier in the absence of methylene blue. This was verified by Ogston and Green who also used a yeast enzyme. The data presented in Table II indicate that Straub's flavoprotein prepared from beef heart muscle functions in the same capacity. In the presence of D-glucose, DPN, and the liver protein, 8 mg. of the flavoprotein catalyzed an oxygen uptake of 535 c.mm. in 120 minutes, whereas 2 mg. of flavoprotein catalyzed an oxygen uptake of 300 c.mm. Since the purified flavoprotein was difficult to prepare, it was decided to use only 2 mg. per vessel in subsequent experiments.

Table III presents data to show the effect of omitting the various factors of the system when heart flavoprotein is the carrier. The factors other than the flavin enzyme were used in the concentrations that had been previously determined to be optimal with the methylene blue system. The

flavoprotein was present at a concentration of 2 mg. per vessel. It is apparent from these results that all of the factors must be present to cause an adequate oxygen uptake. In the absence of D-glucose, DPN, dehydrogenase protein, or flavoprotein the oxygen uptake is between 7 and 14 c.mm. in 120 minutes.

A flavoprotein was prepared from fresh lamb liver according to the method that Straub had developed for the preparation of heart muscle flavoprotein. The liver flavoprotein was used in concentrations from 0.25 to 2.0 mg. per vessel. The latter concentration, the highest concentration tested, catalyzed an oxygen uptake of 87 c.mm. in 120 minutes. Although the activity was low, it is probably significant when compared with the control that contained no flavoprotein. The liver preparation of flavoprotein apparently contained many impurities, since in addition to catalyz-

TABLE III
Omission of Factors in Flavoprotein System

Flavoprotein, 2 mg. in 0.25 ml. of 0.1 M phosphate buffer, pH 7.4; all other constituents and conditions as in Table II.

Vessel pair		1	2	3	4	5
Body	Liver protein Flavoprotein 0.1 M phosphate buffer, pH 7.4	ml.	ml.	ml.	ml.	ml.
		0.25	2.0	2.0	2.0	2.0
Side arm	Water D-Glucose DPN	0.25	0.25	0.25	0.25	0.25
		0.5	0.75	0.75	0.5	0.5
		0.25	0.25	0.25	0.25	0.25
		0.25	0.25	0.25	0.25	0.25
Oxygen uptake	60 min.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.
	120 min.	4	9	8	11	161
		7	13	9	14	304

ing a low oxygen uptake, it was tan in color rather than lemon-yellow, as was the heart flavoprotein.

Effect of Inhibitors and Activators on Flavoprotein System

In order to characterize further the flavoprotein system with respect to the activity of the enzymes involved, it was decided to test the effect of various inhibitors and activators at one concentration each. Table IV summarizes these results. 0.05 M sodium malonate and 3 per cent ethyl urethan were without effect when interpreted on the basis that changes of less than 10 per cent from the water control are not significant. Definite inhibitions were caused by 0.04 M sodium iodoacetate and 100 mg. per cent of atabrine. 0.00002 M catalase produced a rather pronounced activation.

Cytochrome c-Indophenol Oxidase System of Ogston and Green

Since Ogston and Green had been unable to obtain significant activity using the glucose dehydrogenase of Harrison with their own indophenol

oxidase system, it seemed advisable for us to reinvestigate the activity of their oxidase preparation using our purer liver dehydrogenase protein. DPN and D-glucose were added in the amounts previously indicated not to be limiting with 10 mg. of the liver protein. Cytochrome *c* was added in an amount that would insure that this enzyme would not be limiting, namely, 4 mg. per vessel. The indophenol oxidase was used at several concentrations. It is evident from the data presented in Table V that the oxidase is not a significantly active preparation. A small activity is shown when Pairs 2, 3, 4, and 5, containing 1, 0.5, 0.25, and 0.1 ml. of the oxidase, are compared with the oxidaseless control, Pair 7. That this activity is demonstrable 26 hours later is shown by Pair 8. That cytochrome oxi-

TABLE IV

Effect of Various Agents on Flavoprotein System

Body, 10 mg. of liver protein in 1 ml. of 0.1 M phosphate buffer, pH 7.4; 2 mg. of heart flavoprotein in 0.5 ml. of 0.1 M phosphate buffer, pH 7.4; 0.325 ml. of agent in water; 0.925 ml. of 0.1 M phosphate buffer, pH 7.4. Side arm, 0.25 ml. of 2 molal D-glucose in water; DPN, 1 mg. in 0.25 ml. of water. Center well, 0.25 ml. of 10 per cent NaOH. Gas phase, oxygen; temperature, 38°; rate of shaking, 100 per minute.

Final concentration of agent	120 min. oxygen uptake	Deviation from control <i>per cent</i>
	<i>c.mm.</i>	
Water.	322	
0.04 M sodium iodoacetate	215	-33
100 mg. % atabrine	212	-34
Water.	327	
0.05 M sodium malonate	300	-8*
3% ethylurethan	333	+2
0.00002 M catalase	401	+22

* Slight cloudiness.

dase may have been present in small amounts is suggested by the fact that sodium cyanide (Pair 6) produced some inhibition. The relatively large value obtained with the control, containing no oxidase, can be attributed to the 4 mg. of cytochrome *c* per vessel, since in subsequent experiments the same control, containing 1 mg. of cytochrome *c* per vessel, gave a value of 10 c.mm. in 120 minutes.

Cytochrome c-Insoluble Cytochrome Oxidase Complex System

When it became apparent that the preparation of Ogston and Green was ineffective, the three times precipitated oxidase⁶ of Keilin and Hartree, as modified by Hawthorne and Harrison and as modified by us, was next

⁶ Oxidase signifies insoluble cytochrome oxidase complex.

TABLE V

Ogston and Green's Indophenol Oxidase

NaOH, 10 per cent; equal quantities of 1 M NaOH and 1 M NaCN; liver protein, 10 mg. in volume given below of 0.1 M phosphate buffer, pH 7.4; 2 molal D-glucose in water. Gas phase, oxygen; temperature, 38°; rate of shaking, 100 per minute.

Vessel pair.....		1	2	3	4	5	6	7	8
		ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Center well	NaOH	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Body*	NaOH + NaCN								
	Liver protein	1.5	1.5	1.5	1.5	1.5	1.0	1.0	1.0
	0.1 M phosphate buffer, pH 7.4			0.5	0.75	0.9		1.0	
Side arm†	Water	0.25					0.175		0.5
	Indophenol oxidase	1.0	1.0	0.5	0.25	0.1	1.0	0.5	1.0
	10 ⁻⁴ M NaCN						0.325		
D-Glucose			0.25	0.25	0.25	0.25	0.25	0.25	0.25
		c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.
Oxygen uptake	60 min.	8	47	38	34	28	28	26	39
	120 min.	13	82	67	62	54	63	44	81

* 4 mg. of cytochrome c in 0.25 ml. of water added to each vessel.

† 1 mg. of DPN in 0.25 ml. of water added to each vessel.

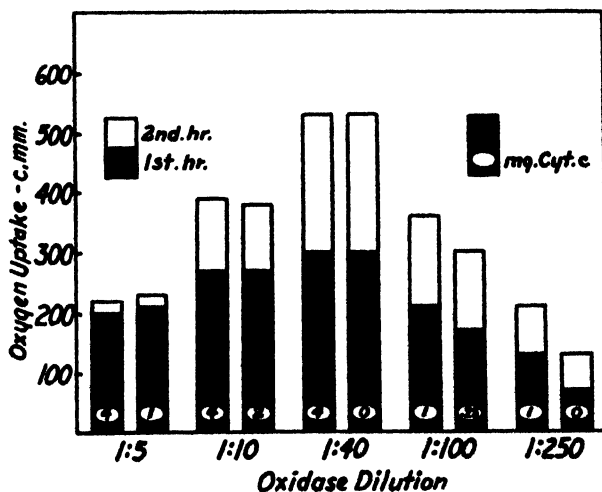


FIG. 1. Body, 10 mg. of liver protein in 1 ml. of 0.1 M phosphate buffer, pH 7.4; 0.5 ml. of 0.1 M phosphate buffer, pH 7.4; cytochrome oxidase complex, stock suspension diluted 1:5, 1:10, 1:40, 1:100, and 1:250 with 0.1 M phosphate buffer, pH 7.4, 1 ml. added to each vessel; above amount of cytochrome c in 0.25 ml. of water. Side arm, 0.25 ml. of 2 molal D-glucose in water; DPN, 1 mg. in 0.25 ml. of water. Center well, 0.25 ml. of 10 per cent NaOH. Gas phase, oxygen; temperature, 38°; rate of shaking, 100 per minute.

tested. The results presented in Fig. 1 show three important effects: (1) that the three times precipitated preparation is extremely active when

tested with d-glucose dehydrogenase and its substrate; (2) that the best activity is obtained at an oxidase dilution of 1:40; (3) that cytochrome *c* becomes limiting only at a dilution of oxidase between 1:40 and 1:100.

When one compares dilutions of the stock oxidase of 1:5, 1:10, 1:40, 1:100, and 1:250, it is apparent that the best activity is obtained at a dilution of 1:40. The oxygen uptake is approximately 535 c.mm. in 120 minutes. Our results differ in this respect from those of Hawthorne and Harrison who obtained their best activity with the stock solution. The oxygen values obtained in the 2nd hour with the 1:5 and 1:10 dilutions of the stock oxidase suggest that the oxidase contained some factor which at the higher concentrations was destroying itself or another enzyme or co-enzyme in the system. The ratio of the oxygen uptake for the 2nd hour when compared to the oxygen uptake for the 1st hour is small when the 1:5 dilution of the oxidase is used, and approaches unity and essentially becomes a constant at the 1:40, 1:100, and 1:250 dilutions.

That cytochrome *c* must be present in the three times precipitated insoluble oxidase complex becomes apparent by comparing the results obtained with the 1:40, 1:100, and 1:250 dilutions of the stock oxidase. Omission of cytochrome *c* from the system with the 1:40 oxidase leaves the oxygen uptake unchanged, whereas with the 1:100 oxidase the substitution of 0.25 mg. of cytochrome *c* for 1 mg. of cytochrome *c* decreases the oxygen uptake from 375 c.mm. in 120 minutes to 300 c.mm. A comparison of the 1:250 oxidase with 1 mg. of cytochrome *c* and with cytochrome *c* omitted shows a 39 per cent decrease in the oxygen uptake.

Effect of Inhibitors and Activators on D-Glucose Dehydrogenase-Insoluble Cytochrome Oxidase Complex System

In Table VI are presented the effects of various inhibitors and activators when each substance was added at only one concentration. Deviations of less than 10 per cent from the water control are not considered significant. 0.00002 M catalase produced a change of only 1 per cent. 0.05 M sodium malonate produced a 10 per cent activation, but since a red precipitate formed when the malonate was added, the effect is to be viewed with caution. 100 mg. per cent of atabrine (also complicated by the formation of a precipitate), 3 per cent ethylurethan, and 0.04 M sodium iodoacetate produced a marked inhibition of the oxygen uptake.

Combined Insoluble Cytochrome Oxidase Complex and Heart Flavoprotein Systems

Since flavoprotein alone could serve as a carrier for the aerobic oxidation of d-glucose in oxygen, it was decided to study the oxidation when added flavoprotein and the cytochromes *c* and oxidase were used together as car-

TABLE VI

Effect of Various Agents on Insoluble Cytochrome Oxidase Complex System

Body, 10 mg. of liver protein in 1 ml. of 0.1 M phosphate buffer, pH 7.4; 1 mg. of cytochrome *c* in 0.25 ml. of water; 1 ml. of 1:100 cytochrome oxidase complex suspension, diluted with 0.1 M phosphate buffer, pH 7.4; 0.175 ml. of 0.1 M phosphate buffer, pH 7.4; 0.325 ml. of agent in water. Side arm, 0.25 ml. of 2 molal D-glucose in water; 1 mg. of DPN in 0.25 ml. of water. Center well, 0.25 ml. of 10 per cent NaOH. Gas phase, oxygen; temperature, 38°; rate of shaking, 100 per minute.

Final concentration of agent	120 min. oxygen uptake	Deviation from control
	<i>c.mm.</i>	<i>per cent</i>
Water.	433	
0.05 M sodium malonate..	478	+10*
100 mg. % atabrine	23	-95†
3% ethylurethan	37	-91
Water.	396	
0.04 M sodium iodoacetate..	36	-91
0.00002 M catalase	400	-1

* Red precipitate at beginning of experiment.

† Precipitate noted at end of experiment.

TABLE VII

Omission of Factors in Combined Insoluble Cytochrome Oxidase Complex and Straub Heart Flavoprotein System

Center well, 0.25 ml. of 10 per cent NaOH; liver protein, 10 mg. in 0.1 M phosphate buffer, pH 7.4; flavoprotein, 2 mg. in 0.1 M phosphate buffer, pH 7.4; cytochrome oxidase complex, stock suspension diluted 1:40 with 0.1 M phosphate buffer, pH 7.4; 2 molal D-glucose in water; DPN, 1 mg. in water. Gas phase, oxygen; temperature, 38°; rate of shaking, 100 per minute.

Vessel pair		1	2	3	4	5	6
		<i>ml.</i>	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>	<i>ml.</i>
Body*	Liver protein		1.0	1.0	1.0	1.0	1.0
	0.1 M phosphate buffer pH 7.4	1.25	0.25	0.5	0.25	1.25	0.25
	Water		0.25		0.25		
	Flavoprotein	0.25	0.25		0.25	0.25	0.25
Side arm	Cytochrome oxidase	1.0	1.0	1.0	1.0		1.0
	D-Glucose	0.25		0.25	0.25	0.25	0.25
	DPN	0.25	0.25	0.25		0.25	0.25
		<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>	<i>c.mm.</i>
Oxygen uptake	60 min.	20	21	319	21	182	322
	120 min.	27	32	586	30	325	591

* Cytochrome *c*, 1 mg. in 0.25 ml. of water added to body of each vessel.

riers. The effect of omitting certain factors of the system containing the oxidase at a 1:40 dilution and with 2 mg. of heart flavoprotein is presented in Table VII. When liver protein, D-glucose, or DPN was omitted, the

oxygen uptake for the 120 minutes was between 27 and 32 c.mm. The complete system consumed 591 c.mm. of oxygen in 120 minutes and the activity was unaffected by omitting the flavoprotein. Omission of the oxidase reduced the oxygen uptake to approximately the value obtained with flavoprotein alone; namely, 325 c.mm. in 120 minutes.

The data in Table VIII present the effects of many factors on the activity of the flavoprotein and insoluble cytochrome complex systems, alone and in combination. The liver protein was always present in the amount of 10 mg. per vessel. The oxidase was used at a dilution of the stock solu-

TABLE VIII

Effect of Air, Oxygen, Cytochrome c, and Cyanide on Two Systems

NaOH, 10 per cent; liver protein, 10 mg. in volume given below of 0.1 M phosphate buffer, pH 7.4; flavoprotein, 2 mg. in volume given below of 0.1 M phosphate buffer, pH 7.4; cytochrome c, 1 mg. in 0.25 ml. of water; cytochrome oxidase complex stock suspension diluted 1:250 with 0.1 M phosphate buffer, pH 7.4. Temperature, 38°; rate of shaking, 100 per minute.

Vessel pair		1	2	3	4	5	6	7	8	9	10	11	12
Gas phase.....		O ₂	Air	Air	Air	Air	Air	Air	Air	Air	Air	O ₂	O ₂
		ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.
Center well	Sodium hydroxide	0.25											
	Water		0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Body*	Liver protein	0.5	0.5	1.0	1.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Flavoprotein				0.5	0.25	0.25		0.25	0.25	0.25	0.25	0.25
	Cytochrome c	0.25	0.25		1.0	0.25	0.25	0.25		0.25	0.25	0.25	0.25
	" oxidase	1.0	1.0	1.0		1.0	1.0	1.0				1.0	1.0
	0.1 M phosphate buffer, pH 7.4	1.0	1.0	0.5		0.75	0.425	0.675	1.75	1.75	1.425	0.75	0.425
	0.01 M sodium cyanide						0.325	0.325			0.325		0.325
	Water			0.25	0.25				0.25				
		c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.	c.mm.
Oxygen uptake	60 min.	112	106	72	110	141	79	11	72	72	74	204	157
	120 min.	197	202	129	208	246	138	14	132	132	136	366	280

* Side arm, 0.25 ml. of 2 molar D-glucose in water and 1 mg. of DPN in 0.25 ml. of water in each vessel.

tion of 1:250 which had previously been found to give submaximal activity with the 10 mg. of liver protein. A comparison of Pairs 1 and 2 indicates that the oxidase system is unaffected by an 80 per cent reduction in the oxygen tension. Earlier experiments had shown that carbon dioxide is not evolved in this system, and, therefore, alkali is not necessary. A comparison of Pairs 2 and 3 shows that cytochrome c is limiting at the 1:250 dilution of the stock oxidase. The addition of flavoprotein (Pair 4) to the system containing no cytochrome c (Pair 3) increases the oxygen uptake. The addition of cytochrome c in the presence of the flavoprotein (Pair 5) increases the oxygen uptake even further to exceed the control (Pair 2) con-

taining no flavoprotein. Sodium cyanide in a final concentration of 0.001 M (Pair 6) inhibits the oxidase system to the extent that the activity of the system with oxidase and flavoprotein (Pair 5) is reduced to the level obtained with flavoprotein alone (Pair 8). The oxidase system alone is inhibited by approximately 90 per cent (compare Pairs 2 and 7). Added cytochrome *c* and sodium cyanide are without effect on the flavoprotein system (Pairs 8, 9, and 10). A comparison of Pairs 11 and 5 shows that the oxygen in air is limiting when the two systems are together, the oxygen uptake being 366 c.mm. and 246 c.mm. in 120 minutes in oxygen and air, respectively. 0.001 M sodium cyanide inhibits the activity of the combined systems in oxygen by approximately 24 per cent (compare Pairs 11 and 12). The value of 280 c.mm. (Pair 12) compares rather favorably with the values obtained with flavoprotein alone, indicating that when the cyanide is added to inhibit the oxidase system the flavoprotein system functions independently.

DISCUSSION

The work of Adler and von Euler (3) and of Ogston and Green (2), which showed that a yeast flavoprotein could mediate the aerobic oxidation of D-glucose, has been verified by using heart flavoprotein as a carrier. It is difficult to explain why such large amounts of flavoprotein were necessary in order to approach the upper limits of dehydrogenase activity. One possibility was that the flavoprotein while being oxidized was producing an amount of hydrogen peroxide sufficient to decrease the rate of the reaction. It was argued that, if this were the case, the addition of catalase should remove the hydrogen peroxide and allow the reaction to approach its true equilibrium. On the other hand, the addition of catalase should also tend to decrease the oxygen uptake, since it would return to the gas phase 1 of 2 oxygen atoms that had been incorporated into the hydrogen peroxide molecule. Since the addition of catalase caused a 22 per cent increase in the oxygen uptake, it seemed that of the two possibilities the accumulation of hydrogen peroxide to inhibit the reaction may have been the dominant one.

The use of the various inhibitors, although only at one concentration each, served further to characterize the enzymes involved in this aerobic oxidation of D-glucose. The failure of sodium malonate to inhibit rules out the succinate-fumarate system as an intermediate or as a coupled reaction in the dehydrogenation. Atabrine at a final concentration of 100 mg. per cent markedly inhibited the flavoprotein system. This substance is known to be a competitive inhibitor of cytochrome reductase (14), a flavoprotein from yeast, and its action presumably is by virtue of its structural similarity to the flavin portion of the prosthetic group of the enzyme. Sodium iodo-

acetate inhibited the flavoprotein system and this strengthens our belief that here, as in other enzyme reactions involving an oxidation, sulfhydryl groups are participating. However, these results are not in agreement with those obtained by Harrison (1), who showed that 0.001 M iodoacetate did not inhibit the anaerobic decolorization of methylene blue by glucose dehydrogenase. It may be that our higher concentration of the inhibitor (0.04 M) had, in part, a non-specific action. On the other hand, it is suggested that the lower concentration used by Harrison may have been insufficient to overcome a protective action exhibited by the other proteins that were present in his less pure preparation.

It has been verified that the three times precipitated insoluble cytochrome oxidase complex of heart muscle, as used by Hawthorne and Harrison (4) and as modified by us, can serve as a carrier with the D-glucose dehydrogenase of lamb liver. The failure of Ogston and Green (2) to verify Harrison's (1) results was probably due to the method they selected for preparing their oxidase. The mortar-ground tissue, instead of being centrifuged, was squeezed through cheese-cloth which undoubtedly allowed many impurities to pass into the preparation. These impurities would not only lower the activity per unit of protein, but might also add to the preparation an enzyme which we suspect to be a ribonuclease and which, therefore, would rapidly destroy the DPN.

A comparison of the results, obtained in air and in oxygen with the cytochrome system alone and with the cytochrome system when flavoprotein is added, suggests that the important physiological system is the cytochrome system of which flavoprotein may be a part. The marked difference in activity, a 50 per cent decrease, that is noted when the flavoprotein system in 100 per cent oxygen is compared with the flavoprotein system in air (20 per cent oxygen) suggests that flavoprotein is an ineffectual carrier at oxygen tensions below 150 mm. This is further borne out by a comparison of the results obtained with the two systems in air and in oxygen when alone and in combination. In 120 minutes the cytochrome system alone in air catalyzed the uptake of 202 c.mm. of oxygen, the flavoprotein system alone used up 132 c.mm., and the two systems together consumed 246 c.mm. The corresponding values in oxygen were 197, 280, and 366 c.mm., respectively. It is apparent that the flavoprotein is of lesser importance as the oxygen tension is reduced. This is in agreement with the results of Green (15).

By omitting each in turn, D-glucose, D-glucose dehydrogenase protein, and DPN have been shown to be necessary constituents of the cytochrome system that aerobically oxidizes D-glucose. That cytochrome *c* is a necessary constituent is evident from the fact that in the more dilute solutions of the stock oxidase (1:100, 1:250) the addition of cytochrome *c* accelerates

the oxygen uptake. Hawthorne and Harrison, even though they were able to show an increase in oxygen uptake under certain conditions, concluded that cytochrome *c* was not absolutely necessary and that in the absence of cytochrome *c* the oxidation of D-glucose was mediated from the dehydrogenase to flavoprotein, to cytochromes *a* and *b*, and finally to cytochrome oxidase. Dewan and Green (16) and Hawthorne and Harrison were not able to demonstrate spectroscopically the presence of cytochrome *c* in their preparations. That our three times precipitated insoluble oxidase complex contains cytochrome *c* is borne out by the following observations: (1) that added cytochrome *c* enhances the activity of the more dilute oxidase preparations; (2) that hydroquinone, whose enzymatic oxidation must be mediated by cytochrome *c* (17, 12), is oxidized by this preparation, and that added cytochrome *c* accelerates the reaction only slightly.⁷

When catalase is added to the cytochrome *c*-insoluble cytochrome oxidase complex system, there is no increase in the oxygen uptake such as was obtained when catalase was added to the flavoprotein system. The marked inhibition obtained when atabrine is added in a final concentration of 100 mg. per cent to the cytochrome system without added flavoprotein must be interpreted with caution, since a precipitate was formed. The inhibition obtained in the presence of 3 per cent ethylurethan (18) suggests that cytochrome *b* may be present and serving as an intermediate. Ethylurethan was without effect when flavoprotein was the sole added carrier. The inhibition observed with sodium cyanide suggests that cytochrome oxidase is one constituent of the carrier system.

The results suggest to us that the aerobic oxidation of D-glucose, when D-glucose dehydrogenase protein and DPN are present, is mediated through the following components of the cytochrome system: cytochrome *c* and cytochrome oxidase. The participation in the cycle of flavoprotein and of cytochromes *a* and *b* has not yet been verified.

SUMMARY

1. It has been demonstrated manometrically that Straub's heart flavoprotein can serve alone as a carrier with a partially purified D-glucose dehydrogenase protein of lamb liver with added diphosphopyridine nucleotide when the gas phase is oxygen. Added crystalline liver catalase increases the oxygen uptake.

2. It has been verified that an insoluble, three times precipitated, heart cytochrome complex, with added cytochrome *c*, can function as a hydrogen carrier with D-glucose dehydrogenase protein of lamb liver and diphosphopyridine nucleotide. Added crystalline catalase is without effect.

⁷ Wainio, W. W., Cooperstein, S. J., Kollen, S., and Eichel, B., unpublished.

3. If Straub's heart flavoprotein is added to the insoluble cytochrome complex system, the flavoprotein can operate as a separate and independent carrier system. When the gas phase is air, the flavoprotein is of lesser importance as an independent system than when the gas phase is oxygen.

4. Cytochrome *c* has been shown to be partly limiting in dilute preparations of a three times precipitated insoluble cytochrome oxidase complex.

5. It has been shown that the enzyme system under consideration here for the aerobic oxidation of D-glucose consists of the following: D-glucose dehydrogenase protein, diphosphopyridine nucleotide, cytochrome *c*, and cytochrome oxidase. The participation in the cycle of flavoprotein and of cytochromes *a* and *b* has not yet been verified.

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STUDIES ON THE DENATURATION OF ANTIBODY

IV. THE INFLUENCE OF pH AND CERTAIN OTHER FACTORS ON THE RATE OF INACTIVATION OF STAPHYLOCOCCUS ANTITOXIN IN UREA SOLUTIONS

BY GEORGE G. WRIGHT AND VERNER SCHOMAKER

(From the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena)*

(Received for publication, January 24, 1948)

In previous work on the denaturation of antibody, studies have been made of some of the factors influencing the inactivation of diphtheria antitoxin in urea solutions (1, 2). A quantitative formulation of a simple kinetic theory was found to fit satisfactorily the experimental data and to offer a reasonable explanation of the deviation of the inactivation from simple first order behavior (2). In the present work we have studied the inactivation of *Staphylococcus* antitoxin, investigating the influence of certain new factors on the rate and course of the over-all reaction, with a view toward gaining further insight into the mechanism of the reactions, and re-investigating the influence of pH, a factor studied previously with diphtheria antitoxin, in order to test the applicability of the proposed kinetic mechanism to different antibodies.

Materials and Methods

The antitoxic globulin was obtained from crude horse plasma¹ by fractionation between 1.33 and 1.68 M ammonium sulfate. The final preparation contained about 9 per cent protein and 260 I.U. of antitoxin per ml. Only the neutralizing power for the *Staphylococcus* α -toxin, which hemolyzes rabbit red blood cells, was considered in the present work. The hemolytic toxin was obtained from a semisolid agar culture of *Staphylococcus aureus* in the usual manner; the toxin was preserved with merthiolate and used in its crude form. The antitoxin assays were carried out in the customary manner as described previously (3). The procedure consists essentially of determining the dilutions of the unknown and of the treated antitoxins required to reduce the toxicity of a constant amount of the toxin, containing about 150 minimal hemolytic units, to a point where only 50 per cent hemolysis of added rabbit red blood cells occurs after standard incubation. The ratio of the dilution of the partially inactivated antitoxin to the dilution of

* Contribution No. 1129.

¹Supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

the untreated antitoxin gives the proportion of antibody activity remaining; micro-Kjeldahl determinations (4) were necessary to correct for the random volume changes which occurred during the dialysis of the samples for the removal of the urea. The over-all determination appears to be accurate to within a few per cent.

RESULTS AND DISCUSSION

Influence of pH on Rate of Inactivation—In the previous work on the kinetics of the inactivation of diphtheria antitoxin in urea it was observed that the pH markedly influenced the course of the reaction. Kinetic experiments were performed with the *Staphylococcus* antitoxin at a comparable series of pH values and in a similar manner. 8 M urea and a protein con-

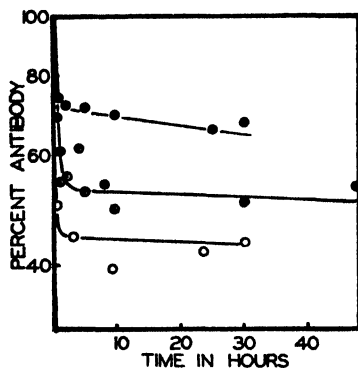


Fig. 1

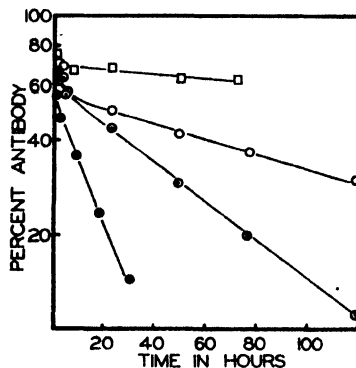


Fig. 2

FIGS. 1 AND 2. Per cent antibody remaining (logarithmic scale) as a function of time of denaturation in 8 M urea at 25°, studied at various pH values. Fig. 1, ○ pH 4.97, ○ pH 5.38, ● pH 6.21; Fig. 2, □ pH 7.40, ○ pH 8.92, ○ pH 9.34, ● pH 9.81. The curves represent the equation $[T] = T_1e^{-\lambda_1 t} + T_2e^{-\lambda_2 t}$. For definitions of symbols see Wright and Schomaker (2).

centration of 0.3 per cent were used; the denaturation reactions were carried out in a thermostat at 25°. The buffer salts were held at an ionic strength of 0.1. Borate buffer was used at pH 9.81, 9.34, and 8.92, phosphate at pH 7.40, and acetate at pH 6.21, 5.38, and 4.97. Samples were removed at the required intervals, diluted with an equal volume of saline, and dialyzed in the cold to remove urea, and the per cent of the original antibody activity was then measured. The experimental results are given in Figs. 1 and 2.

It is clear that the general features of the influence of pH on the inactivation of *Staphylococcus* antitoxin and diphtheria antitoxin are closely similar. Application to the data of the theoretical treatment proposed previously for the inactivation of diphtheria antitoxin in urea solutions gave the curves presented in Figs. 1 and 2; the rate constants derived from the theoretical

curves are given in Table I. This treatment and the proposed kinetic mechanism of which it is the quantitative expression have been developed and discussed in detail previously (2). Briefly, however, the proposed mechanism may be represented by the diagram

where N represents the native, undenatured antibody, I the inactivated antibody, P a hypothetical "protected antibody" which is fully active (or becomes so on removal of urea) but is not susceptible to inactivation, and k_1 , k_2 , and k_3 represent first order rate constants for the reactions as indicated. Examination of Figs. 1 and 2 indicates that the present data are well represented by this treatment, since the experimental points are, in general, closely fitted by the curves. The values of the rate constants de-

TABLE I
Variation with pH of Rate Constants Obtained from Quantitative Treatment Described in Text

pH	k_1	k_2	k_3
	<i>hrs.⁻¹</i>	<i>hrs.⁻¹</i>	<i>hrs.⁻¹</i>
4.97	2.54	2.04	0.0023
5.38	0.83	0.94	0.0020
6.21	0.64	1.62	0.013
7.40	0.46	1.02	0.0049
8.92	0.24	0.30	0.013
9.34	0.30	0.41	0.035
9.81	1.31	1.49	0.10

rived from the theoretical curves and given in Table I, moreover, appear to be identical with those obtained in the experiments with diphtheria antitoxin (2), within the rather large probable errors associated with this sort of curve fitting when the number of experimental points is small.

The satisfactory fit of the experimental data to the theoretical treatment outlined above appears to be additional evidence of the validity of this explanation. If the proposed kinetic explanation is in fact substantially correct, the striking similarity of the values of the calculated rate constants for the two antitoxins suggests that corresponding regions of the protein molecules, with essentially identical major stabilizing bonds, may be involved in the structures of the combining groups of the antibodies for different antigens.

Influence of Neutral Salts on Rate of Inactivation—An interesting aspect of the denaturing action of urea and related substances is the influence of electrolytes on the reaction. The denaturing activity of guanidine salts

was observed to be markedly influenced by the nature of the anion (5). Burk (6) studied the effect of a large number of salts on the concentration of urea necessary for the liberation of sulfhydryl groups of several proteins, and observed very striking differences, the effects varying from strong inhibition of denaturation to striking augmentation. The rate of denaturation of tobacco mosaic virus in urea was also observed to be influenced by the kind and quantity of electrolyte in the solution (7).

Experiments were carried out to determine whether the inactivation of antitoxin is similarly influenced by salts. It was considered possible that the different component reactions of the over-all inactivation might be influenced in different ways, a result which might lead to a separation of the constituent reactions and a better understanding of their nature. Three

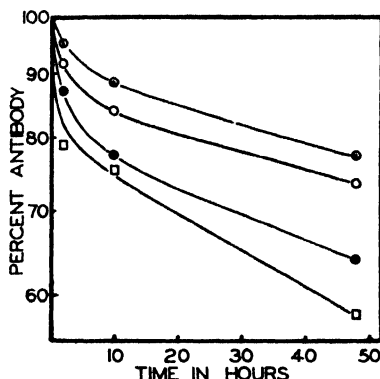


FIG. 3. Per cent antibody remaining (logarithmic scale) as a function of time of denaturation in 6 M urea at 25°, in the presence of 0.25 M concentrations of various salts, as follows: ○ none, ○ sodium sulfate, ● sodium chloride, □ calcium chloride. The curves are empirical.

representative salts were selected from the large group studied by Burk (6). Calcium chloride was chosen as a denaturation-favoring salt, sodium sulfate as a denaturation-inhibiting salt, and sodium chloride as an intermediate salt. The urea concentration was 6 M. Because of the large influence of pH on the rate and course of the over-all reaction, it was necessary to control this factor by the addition of acetate buffer at a final concentration of 0.05 M. The experiments were set up in the usual manner, the different salt solutions being added to measured portions of a solution containing the urea and the buffer, to give a final concentration of 0.25 M. After the solutions had reached temperature equilibrium in the 25° thermostat, the experiment was started by adding the antibody globulin. The samples were removed at intervals, immediately dialyzed, and assayed for antibody activity as before. The results are given in Fig. 3.

Some variation in pH did occur in the experiment, since the pH readings of the solutions were as follows: control 5.66, calcium chloride 5.33, sodium sulfate 5.54, sodium chloride 5.52. These differences appear to be considerably too small to account for the observed effects on the rate of inactivation, however.

It is clear that the influence of the three salts on the inactivation of antibody parallels their influence on the liberation of sulfhydryl groups in other proteins. Thus the addition of calcium chloride promoted the inactivation, sodium sulfate inhibited it, and sodium chloride was intermediate in its effect. There is, however, a slight difference in the behavior of sodium chloride, since it promoted the inactivation slightly, whereas it slightly inhibited the liberation of sulfhydryl groups (6). Thus the results provide additional evidence of the general similarity of the inactivation of antibody to other reactions of proteins which occur in urea solutions and which have been considered classical manifestations of protein denaturation. Although the data are not sufficiently extensive to permit the application of the quantitative treatment of the reaction discussed above, it is clear that a very considerable change in the values of the rate constants in this treatment would be required to produce the large differences in initial slopes among the different experiments. Thus the effects of the salts on the reaction rates may be considerably greater than would appear from a casual inspection of the data for the over-all reaction. It seems as though the three rate constants are affected by the salts in approximately the same manner.

Influence of Hydrostatic Pressure on Rate of Inactivation—It has frequently been observed that the rate of denaturation of proteins is decreased by a moderate increase in hydrostatic pressure, indicating that there is an appreciable volume increase when a protein molecule goes from the normal to the activated state for this reaction. Studies of this sort on the denaturation of the bacterial luminescent system have been particularly complete (8), but evidence has accumulated that denaturation of many other proteins is also accompanied by an increase in volume (9). The rate of denaturation of *Staphylococcus* antitoxin at 65° in dilute salt solutions was observed to decrease with increasing hydrostatic pressure, thus providing additional evidence of the close relationship of antibody inactivation and protein denaturation (3). The influence of hydrostatic pressure on the denaturation of proteins in urea solutions does not appear to have been investigated.

An experiment was performed to determine the influence of hydrostatic pressure on the inactivation of the antitoxin in 6 M urea solutions at 25°. Sodium acetate-sodium chloride buffer was adjusted to give an ionic strength of 0.1 and pH 5.57. As soon as the globulin was added, four 5 ml. samples of the solution were placed in tubes, stoppered with rubber stoppers to leave no air space, placed in the pressure chamber, and a pressure of 10,000 pounds per sq. in. applied. The pressure chamber and the remain-

der of the solution at atmospheric pressure were placed in the 25° thermostat. Samples were taken at intervals from both the atmospheric pressure and high pressure portions, dialyzed, and assayed as before. The results are given in Fig. 4.

Within the experimental error of the method there appears to be no influence of hydrostatic pressure on the rate of the inactivation. In view of the considerable influence of pressure on the rate of inactivation at 65° in the absence of urea, this result was rather unexpected. It seems possible, however, that a reasonable explanation of this difference in behavior may be proposed. The action of urea is generally believed to involve the weakening of the numerous secondary bonds which hold the polypeptide chain in its native configuration (10). High temperature, on the other hand, presumably does not weaken the intramolecular bonds significantly, but breaks

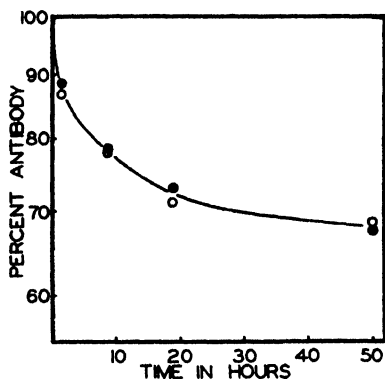


FIG. 4. Per cent antibody remaining (logarithmic scale) as a function of time of denaturation in 6 M urea at 25° at atmospheric pressure (●) and at 10,000 pounds per sq. in. (○). The curve is empirical.

them by means of locally accumulated kinetic energy. The activated state for this latter process might well involve a general volume increase of the protein, whereas the urea-saturated protein, its stabilizing bonds largely neutralized, could unfold without appreciable volume change.

Rate of Change of Viscosity at Various pH Values—The increase in viscosity of serum proteins when subjected to the denaturing action of urea is well known (11). The rate at which this increase occurs, however, does not seem to have been investigated to any considerable extent, although recently the viscosity change has been observed to consist of an initial rapid rise followed by a gradual increase (12). Experiments were undertaken to determine whether the rate of the change in viscosity of the antibody globulin in urea could be correlated in any way with the kinetics of the antibody inactivation.

Solutions at a series of pH values in 8 M urea were studied. These were prepared in the same way as in the antibody inactivation experiments. The antibody globulin was added last, after which the solutions were quickly filtered to remove any trace of lint, and samples were accurately measured into Ostwald viscometers in a thermostat at $25^\circ \pm 0.01^\circ$. Measurements of the flow time were made frequently at first and then at increasing intervals as the viscosity change became slower. Between measurements the viscometers were stoppered to prevent evaporation of the solutions. After completion of each experiment the viscometer was cleaned and dried and the flow time of the solvent was determined on a solution made up in the same way as the denaturation mixture (usually an aliquot of the same solution) except that an equal volume of water was substituted for the antibody

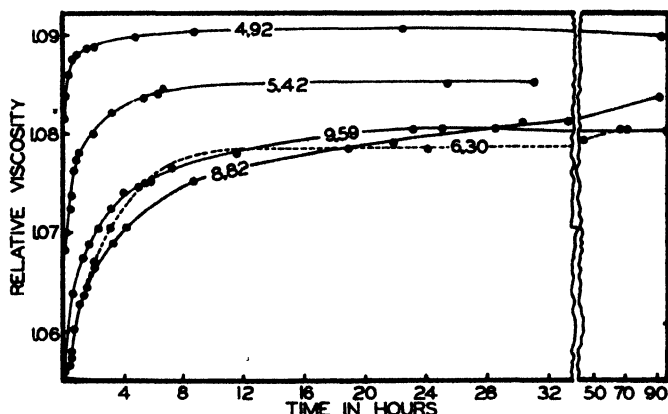


FIG. 5. The relative viscosity of serum globulin as a function of time of denaturation in 8 M urea at 25° , studied at various pH values as indicated. The curves are empirical.

globulin. The relative viscosity was calculated from the two flow times, since density difference between the two solutions was assumed to be negligible. The results of five experiments at a range of pH values are given in Fig. 5. The early points in each experiment represent single measurements of the time of flow, but later measurements are the average of several measurements.

It is clear that the behavior of the viscosity of the solutions varied greatly with the pH and that the results show certain similarities to those of the antibody inactivation experiments. In acid solution the viscosity reached essentially its final value rapidly. At pH 6.30 the initial change was slower but did seem to approach a limiting value. At pH 8.82 the viscosity continued to increase for the duration of the experiment. At pH 9.59 a similar behavior was noted until about 20 hours, when the viscosity leveled off and

finally began to decrease. This suggests that at this high pH some reaction other than the denaturation under consideration, for example a hydrolysis of the peptide bonds, contributed to the change of viscosity. Thus the results are similar to those obtained in the antibody inactivation experiments in certain respects and certainly confirm the existence of relatively slow changes in the protein when exposed to the denaturing action of urea. An adequate test of the applicability to the viscosity changes of the theory proposed for the antibody inactivation (2) would be very difficult to make, however, because it would be necessary to know the viscosities of the different molecular species in the various buffer-urea solutions and to have information about the manner in which their contributions add to produce the observed viscosity.

SUMMARY

A study has been made of the influence of pH, of certain neutral electrolytes, and of hydrostatic pressure on the rate of inactivation of equine *Staphylococcus* antitoxin in urea solutions and of the viscosity changes which take place during the denaturation. Deviations from simple first order behavior similar to those encountered in the inactivation of diphtheria antitoxin were observed, and the course of the reaction could be similarly explained by the simple kinetic mechanism proposed previously, involving two competing reactions of the native protein. The influence of certain electrolytes on the inactivation of the antitoxin was observed to be generally similar to their influence on the liberation by urea of sulfhydryl groups in other proteins. Hydrostatic pressure was found to have little or no effect on the rate or course of the reaction. The rate of the viscosity changes of the protein in the urea solution was also observed to vary with the pH, in a manner which was suggestive of the variation of the inactivation reaction, and confirmed the occurrence of relatively slow changes of the protein in the urea solution. The meaning of the results was discussed.

This investigation was carried out with the aid of a grant from the Rockefeller Foundation. The authors are indebted to Professor Linus Pauling for helpful discussion and to Miss Phyllis Carpenter for technical assistance during part of the work.

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A METHOD FOR THE MEASUREMENT OF RADIOIODINE IN BIOLOGICAL MATERIALS*

By MICHAEL C. BARRY†

(From the Department of Pharmacology, Yale University School of Medicine,
New Haven)

(Received for publication, April 10, 1948)

For the purpose of biochemical studies utilizing radioiodine I^{131} as a tracer material, it is desirable to have a simple, sensitive, and thoroughly validated method for the measurement of the radioactivity of the biological samples. The present investigation was undertaken to provide such a method, with particular reference to the thyroid of the rat, but is adaptable with appropriate modifications to any biological material or compound. In this procedure the iodide in the sample is oxidized to iodate by a modification of the permanganate acid method of Riggs and Man (1), reduced to iodide, and the iodide precipitated as palladium iodide after the addition of a controlled amount of carrier iodide. By this means there is obtained a controlled weight of I^{131} -containing precipitate which is independent of variations in the chemical composition and the amount of the original sample, and which may be easily filtered off for radioactivity measurement.

Materials and Solutions—

Reagent grade analytical chemicals were used throughout the experiments.

Acetone, 5 per cent by volume.

Palladium chloride; 1 ml. of solution contains 10 mg. About 20 ml. of concentrated hydrochloric acid per 250 ml. of solution must be added to dissolve the salt.

Potassium iodide, 40.0 mg. per ml. of solution.

Disodium acid phosphate, anhydrous, approximately 1 mg. per drop of solution.

Sodium acid sulfite, approximately 1 gm. per 50 ml. of solution.

Sulfuric acid, 8 N and 18 N.

Radioactive iodine I^{131} , carrier-free in neutral or weakly basic solution as sodium iodide. Material obtained from the Atomic Energy Commission

* The radioactive iodine used in this investigation was supplied by the Clinton Laboratories and obtained on allocation from the United States Atomic Energy Commission.

This study was aided by grants from the National Institute of Health, the National Research Council, and the National Cancer Institute.

† Fellow of the National Institute of Health. Present address, Sloan-Kettering Institute for Cancer Research, New York 21, New York.

was used without further purification. A stock solution containing approximately 1 microcurie per ml. was prepared by water dilution.

Procedure

Oxidation.—By means of a glass rod the excised rat thyroid is placed in a 10 ml. Pyrex glass-stoppered volumetric flask containing approximately 200 mg. of potassium permanganate and 2 ml. of 18 N sulfuric acid. It is convenient to use a small glass shovel in measuring out the permanganate. The flask is then placed in an asbestos-covered clamp at an inclination of about 45° with the bulb of the flask several mm. above an electric hot-plate. The temperature of the hot-plate is gradually raised until any initial reaction has subsided and steam has begun to issue from the mouth of the flask. Any tendency of the contents to bump out of the flask is counteracted by moving it further above the hot-plate. In this fashion gentle boiling with evaporation of water is accomplished gradually until the volume of liquid has been reduced to one-third or one-half. At this point there will be no tendency to bump, but the reaction should be kept mild in order to prevent loss from spattering. Further increase of temperature is continued until the sulfuric acid begins to reflux within the flask. Shortly after this it should be possible to turn the flask upright and continue refluxing without loss from spattering until a light brown color remains. The flask is then removed from the hot-plate and allowed to cool. The entire oxidation requires from 2 to 3 hours.

Precipitation of Iodide.—When cool, a few ml. of water are added to the flask, followed by several drops of sodium acid sulfite solution, with shaking, until the manganese dioxide has been reduced and the solution is colorless. In the event that it is desired to utilize only a portion of the original sample for radioactivity measurements the solution is now diluted to the mark with water; otherwise no further dilution is made. Then either a suitable aliquot or the entire solution is transferred to a 50 ml. beaker containing a glass stirring rod, several drops of sodium acid sulfite, and 1 ml. of potassium iodide solution. Approximately 1 ml. of acetone is then added, and 3 ml. of palladium chloride solution. If a 1 ml. aliquot of the oxidized solution is taken for radioactivity measurement, the palladium iodide precipitate will be contained in about 25 ml. of solution. After stirring, the precipitate is filtered off under a vacuum on a Pyrex low form, 30 mm. diameter, fritted disk crucible of medium porosity. Three washings of about 25 ml. each, to the second of which are added a few drops of acetone, effect a practically quantitative transfer of the precipitate to the crucible. The sides of the crucible are then washed down successively with 5 per cent acetone, water, and pure acetone. With this technique very little if any palladium iodide will remain on the sides of the crucibles.

Measurement of Radioactivity—After drying at 110° for a few hours the crucibles are ready for measurement. In these experiments the measuring equipment consisted of a Radiation Counter Laboratories, mark 6, model 3, thin wall (30 mg. per sq. cm.) β -counter assembly in conjunction with an automatic recording counting rate meter and calibrating multivibrator, as described by Kip and associates (2) and by Mauro and Barry (3). The background counting rate was approximately 50 counts per minute. Care was taken to adjust the heights of the crucibles with aluminum disks so that all the palladium iodide precipitates would be approximately the same distance from the counter.

EXPERIMENTAL

Reproducibility—As a test of the reproducibility of the method several groups of six or more crucibles were prepared by adding the same amount

TABLE I
Reproducibility of Method

Experiment No.	No. of crucibles	Coefficient of variation
		<i>per cent</i>
1	6	1.6
2	6	2.6
3	6	1.8
4	6	1.9
5	12	1.4
6	6	3.5
7	6	3.3

of aqueous I^{131} with a 1 ml. pipette or tuberculin syringe to 50 ml. beakers containing several drops of sodium acid sulfite and 8 N sulfuric acid. Potassium iodide and palladium chloride were added and the precipitates filtered off and measured as described above, with the exception that in some of the experiments acetone was omitted. The results are summarized in Table I. In Experiment 1, acetone was not used. Experiments 2 and 3 were re-measurements of the crucibles in Experiment 1. The I^{131} in Experiment 4 was added to 10 ml. volumetric flasks containing the solution resulting from the oxidation of approximately 20 mg. of rat tissue. After diluting to 10 ml. with water, 1 ml. aliquots from each were treated as above. Experiment 5 consisted of the simultaneous measurement of the crucibles in Experiments 1 and 4. In Experiments 6 and 7 a 1 ml. tuberculin syringe instead of a pipette was used to deliver the I^{131} , and acetone was added.

Half Life of I^{131} —Remeasurement of the same crucible after several weeks provided a value for the half life of I^{131} differing by 4.3 per cent from the

accepted value of 8.0 days. Thus there would not appear to be any change in activity of the palladium iodide unexplained by radioactive decay.

Linearity of Dilution—Triplicate samples at each of three I^{131} dilutions were prepared as in the reproducibility measurements above without the use of acetone. The results are presented in Table II. The activity of the most dilute sample was about 5 times the background.

Effect of Thickness of Palladium Iodide Precipitate on Measured Activity—Three sets of crucibles containing equal amounts of I^{131} were prepared with palladium iodide thicknesses of 6.2, 12.4, and 18.6 mg. per sq. cm. The activities of the two latter were respectively 5 and 15 per cent lower than those of the 6.2 mg. per sq. cm. thickness. A thickness of 6.2 mg. per sq. cm. of palladium iodide was that obtained by the precipitation of the iodide in 40 mg. of potassium iodide and its uniform deposition on a 30 mm. diameter fritted disk.

TABLE II
Dilution Linearity

Dilution	Activity		Deviation from theoretical per cent
	Experimental counts per min	Calculated counts per min	
1	3420	(3420)*	
0.2	674	684	-1.6
0.08	271	274	-0.7

* Experimentally determined.

Recovery of Palladium Iodide Gravimetrically—In four experiments consisting respectively of nine, six, six, and twelve crucibles each the weights of the palladium iodide precipitates were determined. In Experiments 1 and 3 the precipitates were prepared as in the reproducibility measurements. Acetone was not used in Experiment 1. Experiment 4 crucibles were the precipitates from a group of rat thyroids prepared according to the procedure given earlier. The average precipitate weight was 43.60 ± 0.17 mg.; as tested by the analysis of variance there was no significant difference in weights between the four experiments. On the assumption that 40.0 mg. of potassium iodide were added in each precipitation, 43.4 mg. of palladium iodide would have resulted. The average weight obtained, 43.6 mg., corresponds to the addition of 40.15 mg. of potassium iodide. This deviation is within the possible error in weighing and in the delivery of 1 ml. of solution by volumetric pipette.

Recovery of Radioiodine—Loss of I^{131} during oxidation was measured by a

series of three experiments, in each of which the activity of six samples prepared as in the reproducibility measurements was compared with a set of six containing originally the same amount of radioiodine but oxidized with 10 to 25 mg. of non-radioactive rat tissue. In the preparation of these latter samples the I^{131} was added to the 10 ml. oxidizing flasks, the sides rinsed with 2 to 3 ml. of water, about 5 mg. of disodium hydrogen phosphate were added, and the solution evaporated until only a few drops remained. Then potassium permanganate, sulfuric acid, and rat tissue were added and the determination conducted according to the procedure given earlier. In the case of both groups acetone was omitted from the filtration of the palladium iodide precipitate. The average recovery was 99.5 per cent. As tested by an analysis of variance the difference between the oxidized and non-oxidized groups was not significant. On the assumption of the same variance a mean difference of about 2 per cent would have

TABLE III
Recovery of Radioiodine

Experiment No.	Scale readings							Per cent recovery
1	Oxidized	60	59	57	59	56	58	99.4
	Non-oxidized	60	60	58	59	58	56	
2	Oxidized	73	70	71	71	67	68	98.5
	Non-oxidized	72	73	70	70	70	71	
3	Oxidized	47	48	46	47	45	49	100.2
	Non-oxidized	48	47	45	48	45	44	

been sufficient to produce a significant result. The data are presented in Table III. Readings are in arbitrary scale units.

Efficiency of Radioactivity Measurement—An I^{131} sample, whose absolute disintegration rate had been determined with a National Bureau of Standards radium D + E β -ray standard, was precipitated and measured as in the reproducibility experiments. In this way the efficiency of this method was found to be about 1 per cent. On the basis of sample counter geometry an efficiency of approximately 3 per cent would have been expected. The additional loss is due principally to β -ray absorption by the relatively thick counter window (30 mg. per sq. cm.).

Palladium Iodide on Walls of Crucibles—When acetone was omitted from the filtration step, a small amount of palladium iodide remained on the walls of the crucibles. In the case of eleven crucibles, judged visually to have an average to maximum amount of precipitate on the walls, the weight of precipitate was measured and found to vary between 0.1 and 0.9 mg.

Removal of the precipitate resulted in reductions of radioactivity varying from 0 to 2 per cent.

DISCUSSION

Under the conditions described this method is suitable for the oxidation and measurement of the I^{131} content of thyroids from normal rats weighing as much as 100 gm. and having fresh thyroid weights of approximately 30 mg. Larger amounts of material could be handled by either or both of the two ways following. First it would be very simple to increase considerably the efficiency of the radioactivity measurements. With the present technique it was found that about 1 per cent of the disintegrating atoms of I^{131} was actually detected. If we assume conservatively that a counting rate of about twice the background, 100 counts per minute, would be required for reasonably precise measurement of activity, it would be possible to measure 0.17 millirutherford of I^{131} (or less accurately stated, 4.5×10^{-3} microcurie). By adopting the precipitation technique described by Henriques *et al.* (4), and finally mounting the palladium iodide precipitate on a 20 mm. filter paper about 2 mm. from a thin window, lead-shielded, bell-type counter with a background of 30 to 40 counts per minute, at least 30 per cent of the I^{131} disintegrations could be detected. This would make possible the measurement of 4×10^{-3} millirutherford (10^{-4} microcurie), a considerable improvement in sensitivity. This sensitivity could be utilized to reduce the amount of material required, 10 to 25 mg. aliquots being taken for analysis.

Secondly, modification of the oxidation conditions could be made to accommodate larger amounts of organic matter. An approximately 10-fold increase in weight of oxidized material probably could be made with a proportionate increase in quantities of reagents and size of oxidation flasks. Obviously, increasing the amount of tracer would further increase the dilution possible, with a consequent reduction in amount of necessary material and required sensitivity.

SUMMARY

1. A convenient method for measuring radioactive iodine, I^{131} , in the normal rat thyroid is described.
2. With the technique described a sensitivity of 0.17 millirutherford is achieved. An ultimate sensitivity of at least 4×10^{-3} millirutherford, 10^{-4} microcurie, is possible.
3. The method is readily adaptable to other biological materials.
4. Recoveries of added radioiodine are 99 per cent.

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ESTIMATION OF α -AMINO ACIDS IN PURE SOLUTIONS, IN BLOOD, AND IN URINE WITH *peri*-NAPHTHINDAN-2,3,4-TRIONE HYDRATE

By RADWAN MOUBASHER

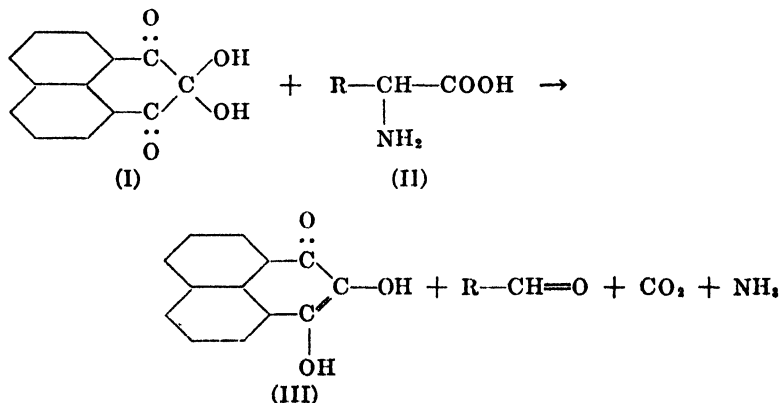
(From the Faculty of Science, Fouad Ist University, Cairo, Egypt)

(Received for publication, January 23, 1948)

Ruhemann (1) reported that when free amino acids are warmed in slightly acid or neutral solution with triketohydrindene hydrate (ninhydrin) they are decomposed to yield carbon dioxide, ammonia, and the next lower aldehyde. Glycine is an exception to this rule and does not yield formaldehyde.

Van Slyke, MacFadyen, and Hamilton (2) have described a method for the determination of free amino acids by titration of the carbon dioxide formed in the reaction with ninhydrin; Van Slyke, Dillon, MacFadyen, and Hamilton (3) have pointed out that isatin in glacial acetic acid and chloramine-T, at pH 2.5, may be used in place of ninhydrin. Virtanen, Laine, and Toivonen (4) have described a quantitative method for the estimation of amino acids in protein hydrolysates based on the quantity of aldehyde liberated by means of ninhydrin.

In the present work it is shown that *peri*-naphthindan-2,3,4-trione hydrate quantitatively decomposes α -amino acids in slightly acid medium to



the corresponding aldehyde with 1 carbon atom less (cf. Schönberg, Moubasher, and Mostafa (5)).

peri-Naphthindan-2,3,4-trione hydrate is valuable for the determination of free α -amino acids in blood and urine by the estimation of the aldehydes formed during the reaction. In the case of blood the proteins can be

removed without loss of amino acids. Picric acid has been found to be the most convenient reagent for this purpose (*cf.* Hamilton and Van Slyke (6)). Its application is very simple and yields a filtrate of pH 1.8 to 2.0, which is suitable without the addition of any other buffer for the *peri*-naphthindan-2,3,4-trione hydrate reaction.

Unlike the *peri*-naphthindan-2,3,4-trione hydrate method, the nitrous acid method applied to urine by Van Slyke and Kirk (7) and the formol titration of Sørensen (8) can be used to estimate not only the α -amino nitrogen of free amino acids but also the terminal amino groups of peptides, as well as amino groups of purines, pyrimidines, and aliphatic amines other than amino acids.

Preparation of peri-Naphthindan-2,3,4-trione Hydrate—*peri*-Naphthindan-2,3,4-trione hydrate is easily prepared (*cf.* Errera (9)) by treating hydroxyketoperinaphthindene with phenylhydrazine in acetic acid solution and boiling under a reflux condenser for 10 minutes. The intense red crystalline substance obtained is filtered off and treated with bromine water until the red color changes to yellow and the excess bromine is driven off by boiling for a short time; on cooling, golden yellow prisms of *peri*-naphthindan-2,3,4-trione hydrate separate out. It is soluble in water, alcohol, acetic acid, slightly soluble in benzene, and more soluble in xylene. It is stable at 100°, but at 110° it gradually loses water and passes into the anhydrous triketone, melting at 273° with decomposition. It gives an intense blue coloration with strong alkali, reduces Fehling's solution and silver salts, and is reduced by phenylhydrazine, hydroxylamine, sulfur dioxide, and hydrogen sulfide. For other properties, *cf.* (10, 11).

In addition to the ease of its preparation, *peri*-naphthindan-2,3,4-trione hydrate is relatively inexpensive as compared with ninhydrin. Moreover, the reduction product obtained in the case of ninhydrin cannot be regenerated and reoxidized to reform ninhydrin, while in the case of *peri*-naphthindan-2,3,4-trione hydrate (I), its reduction product, dihydroxyketoperinaphthindene (III), can be separated and reoxidized by bromine water to the initial substance almost quantitatively.

Estimation of α -Amino Acids in Pure Solutions

Principle—The estimation of the following α -amino acids has been carried out with *peri*-naphthindan-2,3,4-trione hydrate, as they are converted to the next corresponding volatile aldehyde with 1 carbon atom less when they are warmed in a dilute buffer solution: alanine, leucine, isoleucine, valine, serine, aspartic acid, and phenylaminoacetic acid. Aspartic acid is decomposed to acetaldehyde (proved by the formation of the 2,4-dinitrophenylhydrazone derivative).

Reagents—

1. Citrate buffers (3); pH 4.7, grind together 17.65 gm. of $\text{Na}_2\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ and 8.4 gm. of $\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$ to a fine powder. pH 2.5, grind together 2.06 gm. of $\text{Na}_2\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ and 19.15 gm. of $\text{C}_6\text{H}_5\text{O}_7 \cdot \text{H}_2\text{O}$.
2. Sodium bisulfite solution, 1 per cent.
3. Sodium bisulfite, 0.01 N.
4. Iodine, 0.01 N.
5. Saturated solution of sodium bicarbonate.
6. Starch solution, freshly prepared.
7. Pure *peri*-naphthindan-2,3,4-trione hydrate.

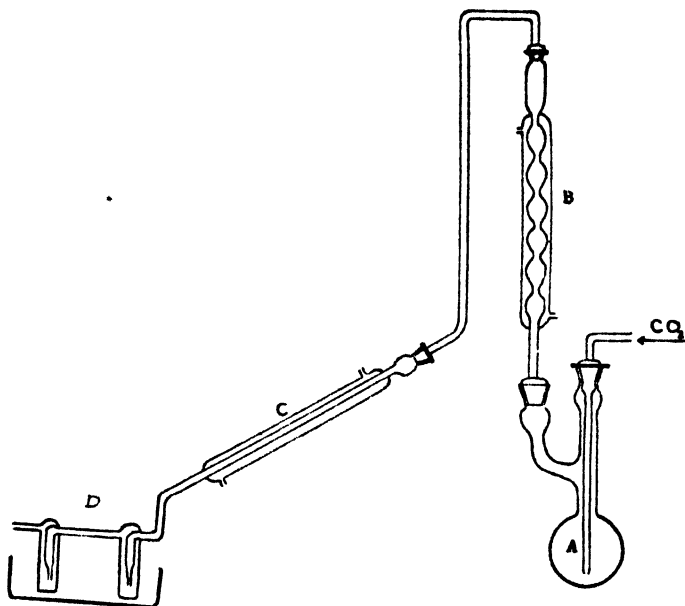


FIG. 1. Reaction and distilling apparatus

Apparatus—

1. Rehberg micro burette of 2 cc. capacity.
2. Micro pipette of 2 cc. capacity.
3. Reaction and distilling apparatus. A distillation flask (A) is provided with a capillary tube for the passage of carbon dioxide, and an efficient reflux condenser (B) is connected by means of glass tubing to a Liebig condenser (C). The receiver is formed by two washing flasks cooled in ice (D) (Fig. 1).

Method

0.2 to 5.0 mg. of the amino acid (which must be dried over sulfuric acid in a vacuum desiccator overnight to constant weight) in 20 cc. of distilled

water and 50 mg. of the appropriate citrate buffer are placed in the reaction vessel with 50 mg. of the pulverized *peri*-naphthindan-2,3,4-trione hydrate. A continual current of carbon dioxide is allowed to pass through the apparatus during heating. The distillate is trapped in 5 cc. of 1 per cent aqueous sodium bisulfite placed in special bubblers which are cooled externally with ice. The reaction vessel is heated in an oil bath at 140–150° for 50 minutes. The contents of the receivers are transferred quantita-

TABLE I

Reaction of Amino Acids with peri-Naphthindan-2,3,4-trione Hydrate

1 cc. of 0.01 N I₂ corresponds to 0.445 mg. of alanine, 0.655 mg. of leucine or isoleucine, 0.585 mg. of valine, 0.525 mg. of serine, 0.665 mg. of aspartic acid, and 0.755 mg. of phenylaminoacetic acid.

Amino acid	Amount	pH	0.01 N I ₂	Recovery
	mg.		cc	per cent
Alanine	3	2.5	6.6	97.9
	3	4.7	6.6	97.9
	0	2.5	0.0	0.0
Leucine	3	2.5	4.5	98.2
	3	4.7	4.5	98.2
	0	2.5	0.0	0.0
Isoleucine	3	2.5	4.5	98.2
	3	4.7	4.5	98.2
	0	2.5	0.0	0.0
Valine	5.2	2.5	8.8	99.0
	5.2	4.7	8.7	97.8
	0	2.5	0.0	0.0
Serine	3	2.5	5.5	96.2
	3	4.7	5.5	96.2
	0	2.5	0.0	0.0
Aspartic acid	3	2.5	4.4	97.5
	3	4.7	4.4	97.5
	0	2.5	0.0	0.0
Phenylaminoacetic acid	4	2.5	5.1	96.2
	4	4.7	5.1	96.2
	0	2.5	0.0	0.0

tively to an Erlenmeyer flask and the excess of sodium bisulfite is removed with 0.01 N iodine, and then the slight excess of iodine is destroyed with a minimal quantity of 0.01 N sodium bisulfite, freshly prepared starch solution being used as an internal indicator. The bound sodium bisulfite is liberated by the addition of 20 cc. of a saturated solution of sodium bicarbonate. The free sodium bisulfite is then titrated by means of 0.01 N iodine. The data in Table I give the results obtained with several amino acids.

Estimation of α -Amino Acids in Blood—1 cc. of blood provides sufficient

material for analysis. It is pipetted into a round bottom centrifuge tube and 5 cc. of 1 per cent picric acid solution are added. The mixture is shaken vigorously and complete precipitation of the protein is effected in a few seconds. Filtration is carried out by means of a sintered glass micro filter and the precipitate is washed several times with a very small amount of water. The filtrate (about 20 cc.) is placed in the reaction vessel, and 15 cc. of distilled water are added with 20 mg. of the powdered *peri-naphthindan-2,3,4-trione* hydrate; a continual current of carbon dioxide is allowed to pass through the apparatus during heating. The distillate is trapped in 5 cc. of 0.5 per cent aqueous sodium bisulfite in the receiver which is cooled with ice. The reaction vessel is heated in an oil bath at 140–150° for 30 minutes. The contents of the receiver are transferred quantitatively and the bound sodium bisulfite is liberated and titrated as in the case of the solutions of amino acids. The results are presented in Table II.

TABLE II
Determination of Amino Acids in Blood

Amount of blood	0.01 N I ₂	Amino acid nitrogen
cc.	cc.	mg. per cent
1	0.8	5.6
1	0.74	5.18
1.5	1.14	7.98
1.5	1.2	8.4
1 + 1 mg. alanine	2.8	19.6

Estimation of α -Amino Acids in Urine—If the analysis cannot be started within an hour or two after the urine is collected, it is saturated with thymol and stored in a refrigerator. 2 cc. of urine are placed in the reaction vessel as described previously with 1 drop of 0.04 per cent bromocresol green, and 5 N sulfuric acid is added until the solution is just yellow (approximately pH 3); then 100 mg. of citrate buffer of pH 2.5 and 100 mg. of powdered *peri-naphthindan-2,3,4-trione* hydrate are added and the mixture is heated in an oil bath at 150° for 20 minutes. The distillate is collected and the amount of the bound sodium bisulfite is estimated as mentioned above. The results obtained are summarized in Table III.

Action of peri-Naphthindan-2,3,4-trione Hydrate on Aspartic Acid—For this experiment the apparatus described in Fig. 1 is modified so as to replace the bubblers with an Erlenmeyer flask which contains an alcoholic solution of 2,4-dinitrophenylhydrazine. *peri-Naphthindan-2,3,4-trione* hydrate (0.5 gm.), aspartic acid (0.29 gm.), and 100 cc. of water are placed in the Claisen flask fitted with the condenser; a continual current of carbon dioxide

is allowed to pass through the apparatus and the receiver which contains 0.3 gm. of 2,4-dinitrophenylhydrazine in alcohol (20 cc.). The mixture in the flask is boiled for about 20 minutes, after which orange-yellow needles are obtained in the receiver; the determination of the melting point (167°) and the mixed melting point proved the compound to be acetaldehyde-2,4-dinitrophenylhydrazone.

$C_8H_8O_4N_4$. Calculated. C 42.9, H 3.5, N 25.0
Found. " 43.2, " 3.5, " 24.9

Regeneration of peri-Naphthindan-2,3,4-trione Hydrate (I)—The reaction product obtained by the action of the amino acid on *peri*-naphthindan-2,3,4-trione hydrate, which consists of dihydroxy*perinaphthindene* (III), is filtered off and washed with hot water and then treated with dilute bromine water with shaking. The intense red color disappears, with the formation of an almost colorless substance. The whole mixture is concentrated and

TABLE III
Determination of Amino Acids in Urine

Amount of urine	0.01 N I_2	Amino acid nitrogen
cc.	cc.	gm. per cent
1	6.0	0.042
1	5.8	0.040
1.5	8.6	0.0602
1.5	8.6	0.0602
1 + 1 mg. alanine	8.0	0.056

left to cool, whereupon *peri*-naphthindan-2,3,4-trione hydrate separates as a yellowish crystalline precipitate, as shown by determinations of melting point, mixed melting point, and color test with sodium hydroxide solution.

SUMMARY

1. *peri*-Naphthindan-2,3,4-trione hydrate can be used for the determination of α -amino acids, as they are decomposed by this reagent quantitatively to the next lower corresponding aldehyde, under the same experimental conditions as with ninhydrin. The new reagent has the advantage of being less expensive and can be regenerated almost quantitatively.

2. α -Amino acids can be estimated in blood and urine with the same reagent under conditions similar to those used in solutions of amino acids.

3. Aspartic acid is decomposed by *peri*-naphthindan-2,3,4-trione hydrate to give acetaldehyde, which was identified by means of its 2,4-dinitrophenylhydrazone derivative.

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THE INHIBITION BY STREPTOMYCIN OF ADAPTIVE ENZYME FORMATION IN MYCOBACTERIA*

By ROBERT J. FITZGERALD, FREDERICK BERNHEIM, AND DOROTHEA B. FITZGERALD

(From the Department of Physiology and Pharmacology, Duke University School of Medicine, Durham, North Carolina)

(Received for publication, April 19, 1948)

A number of *Mycobacteria* oxidize benzoic acid and some can use it as the sole source of carbon (1). Upon addition of benzoic acid to suspensions of washed organisms in the Warburg vessel its oxidation proceeds slowly for 30 to 60 minutes, then very rapidly until about half the amount of oxygen for complete combustion is taken up. The rate then parallels that of the autorepiration. It was thought that this latent period was due to the slow penetration of benzoic acid into the cell. Cells grown with benzoic acid in the medium show, however, a much shorter latent period, and Stanier (2) has presented evidence that benzoic acid and related compounds are oxidized by *Pseudomonas fluorescens* after a similar latent period which he attributes to the formation in the non-proliferating cell suspension of the appropriate adaptive enzymes. We have previously shown (1) that streptomycin inhibits the oxidation of benzoic acid by *Mycobacteria*. This effect might be the result of the inhibition of the benzoic acid oxidase or the inhibition of the formation of the enzyme by the drug. Evidence indicates (3) that the latter explanation is correct. The following contains further quantitative data on the formation of an adaptive enzyme and the effect of streptomycin on this process.

EXPERIMENTAL

Most of the experiments were done with two organisms, *Mycobacterium tuberculosis*, BCG strain (American Type Culture Collection 8240), and *Mycobacterium lacticola* (obtained originally from Dr. Van Niel's collection). The BCG strain was grown 72 hours in Long's synthetic medium, ground and washed with distilled water, and finally suspended in 0.05 M sodium-potassium phosphate buffer, pH 6.7, so that 1.0 cc. contained 0.1 cc. of the packed bacteria. Each Warburg vessel contained 0.5 cc. of this bacterial suspension in a final volume of 2.0 cc. *Mycobacterium lacticola* was grown for 48 hours in the synthetic medium of Kohn and Harris (4) and the inoculum prepared in like manner.

* Aided by a grant from the Duke University Research Council.

The first experiments were done with *Mycobacterium lacticola* which is the only one of several *Mycobacteria* tested which will oxidize *p*- and *m*-hydroxybenzoic acids as well as benzoic acid itself. The effect of streptomycin was tested on the oxidation of *p*-hydroxybenzoic acid by the normal bacteria and by those grown in a medium containing 40 mg. per cent of *p*-hydroxybenzoate. Fig. 1 shows that increasing concentrations of streptomycin cause greater inhibitions of the oxidation of *p*-hydroxybenzoic

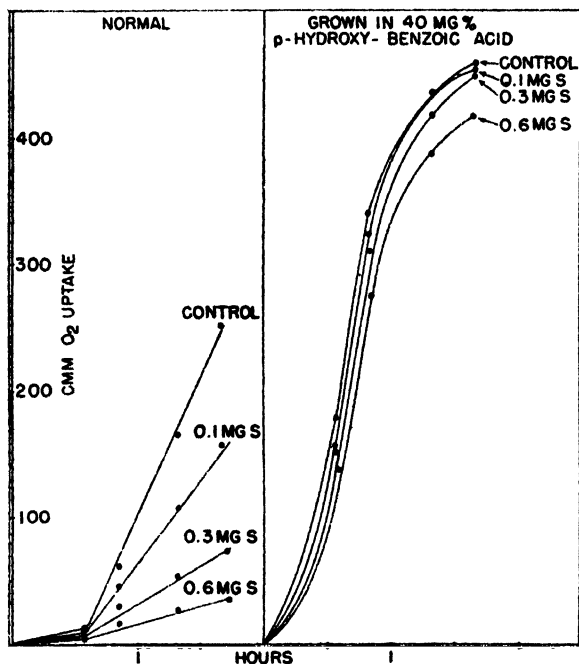


FIG. 1. The effect of streptomycin on the oxidation of *p*-hydroxybenzoic acid by *Mycobacterium lacticola* grown in a normal medium and in the presence of 40 mg. per cent of *p*-hydroxybenzoic acid. pH 6.7; temperature 37°. The control oxygen uptakes have been subtracted in each case.

acid by the normal cells but have comparatively little effect on cells in which the adaptive enzyme has already been formed.

With this species of *Mycobacteria* it is possible to test the specificity of the adaptive enzyme production; in other words, the effect that growth in benzoic acid will have on the subsequent oxidation of *p*-hydroxybenzoic acid and vice versa. Table I shows that growth in benzoic acid increases to a small extent the amount of adaptive enzyme for *p*-hydroxybenzoic acid and that growth in *p*-hydroxybenzoic acid has a somewhat greater effect on the amount of adaptive enzyme for benzoic acid and that in-

hibition of this reaction by streptomycin is correspondingly less. It is also quite clear that the two acids are oxidized by separate enzymes. Similar experiments can be done with *m*-hydroxybenzoic acid, indicating that it also is oxidized by a specific enzyme.

The next question of interest was to determine the amount of benzoic acid required by the cell suspension to produce a measurable amount of adaptive enzyme and the time of exposure necessary for the effect to occur. For these experiments *Mycobacterium tuberculosis* BCG was used, since it is much more sensitive to streptomycin than *Mycobacterium lacticola*. Suspensions were incubated in the Warburg vessel at 37° for various periods

TABLE I

Effect of Growing Mycobacterium lacticola with and without Benzoate or p-Hydroxybenzoate on Subsequent Rate of Oxidation of 1.0 Mg. of Each and Effect of Streptomycin Thereon

The control uptakes have been subtracted. N. = normal cells; B. = cells grown 48 hours in 40 mg. per cent of benzoate; P. = cells grown 48 hours in 40 mg. per cent of *p*-hydroxybenzoate. The figures represent c.mm. of oxygen uptake. pH 6.7; temperature 37°.

Time, hr., min	N.	B.	N.	B.	Time, hr., min.	N.	N.	Inhi- bition	P.	P.	Inhi- bition
	Ben- zoate	Ben- zoate	<i>p</i> -Hy- droxy- benzoate	<i>p</i> -Hy- droxy- benzoate		Ben- zoate	Ben- zoate + 0.1 mg. strepto- mycin		Ben- zoate	Ben- zoate + 0.1 mg. strepto- mycin	
								per cent			per cent
0 30	-6	93	0	4	0 30	-10	-2		-20	-10	
1 00	4	276	62	85	1 00	-10	-18		-14	-8	
1 30	59	427	186	224	1 30	13	-13		52	15	
2 00	139	554	338	399	2 00	67	0		193	48	75
2 30	229	563	454	508	2 30	132	12	91	306	84	73
3 00	358	577	553	579	3 00	330	50	85	520	182	65
					4 00	439	81	84	539	264	51

with 5.0 to 10.0 γ of sodium benzoate and then 1.0 mg. of sodium benzoate with or without 10 to 20 γ of streptomycin was added from the side arms. Table II shows that 15 minutes incubation with 5 γ of benzoate are sufficient for the formation of a demonstrable amount of adaptive enzyme, as shown by the shorter latent period before oxidation occurs and the decreased inhibition by streptomycin. The effect of 10 γ of benzoate is correspondingly greater, but 20 γ are little better than 10 γ , presumably because time rather than concentration has become the limiting factor.

In order to eliminate the time factor, various concentrations of benzoate were incubated with the cell suspensions for 60 minutes prior to the addi-

tion of the 1.0 mg. of sodium benzoate. Preincubation with 100 γ of benzoate for this length of time evokes almost the maximum amount of adaptive enzymes shown in Table III by the small inhibition by streptomycin. This value is not absolute but varies with the number of bacteria and the concentration of streptomycin relative to this number.

Tables II and III show that maximum inhibition by streptomycin does not occur immediately. This latent period is probably the result of the relatively slow penetration of the drug into the cell because when streptomycin is added 90 minutes before the benzoate maximum inhibition occurs

TABLE II

Effect of Preincubation with 5.0 and 10.0 γ of Sodium Benzoate with Suspensions of Mycobacterium tuberculosis BCG for 15 Minutes on Subsequent Oxidation of 1.0 Mg. of Sodium Benzoate and Effect of 20 γ of Streptomycin Thereon

The control uptakes have been subtracted. The figures, in c.mm. of O_2 , represent the oxidation of the 1.0 mg. of benzoate. pH 6.7; temperature 37°.

Time, hrs., min.	Control			Preincubation with 5.0 γ benzoate			Preincubation with 10.0 γ benzoate		
	Benzoate alone	Benzoate + strepto- mycin	Inhibition	Benzoate alone	Benzoate + strepto- mycin	Inhibition	Benzoate alone	Benzoate + strepto- mycin	Inhibition
			per cent			per cent			per cent
0 15	2	4	0	11	9	0	8	11	0
0 30	12	11	0	26	24	0	25	29	0
0 45	26	20	23	53	41	23	59	54	8
1 00	50	29	42	87	60	31	97	79	19
1 15	87	37	57	148	84	42	162	112	31
1 30	135	53	61	205	98	52	229	134	41
1 45	204	57	72	279	119	57	305	161	47
2 00	275	64	77	356	132	63	392	181	54
2 15	360	70	81	436	149	66	452	208	54
2 30	433	77	82	486	164	66	480	228	53
3 30	465	92	81	513	203	62	506	287	44

immediately. Thus the drug inhibits the formation of the benzoic acid oxidase as well as other enzymes involved in the degradation of the benzoic acid molecule.

Another approach to the problem can be obtained by the use of preparations of sonically ruptured cells. A Raytheon oscillator with a frequency of 9000 cycles per second and an average output of 60 watts was used. Cultures of *Mycobacterium lacticola* grown in normal media and in *m*-hydroxybenzoic acid as the sole carbon source were washed by centrifugation and placed in the oscillator for 20 minutes. In this length of time 90 per cent of the bacteria was killed as indicated by plate counts and some

of the enzymatic activity had been lost. Increasing the time of shaking further reduced the enzymatic activity. Table IV shows that this treatment greatly reduces the oxidation of *m*-hydroxybenzoic acid by the normal

TABLE III

Effect of Preincubation of Various Amounts of Sodium Benzoate for 60 minutes on Subsequent Oxidation of 1.0 mg. of Sodium Benzoate by Mycobacterium tuberculosis BCG and Effect of 20 γ of Streptomycin Thereon

The control uptakes have been subtracted. The figures represent c.mm. of oxygen uptake. pH 6.7; temperature 37°.

Time, hrs., min.	Control			Preincubation with 50- γ benzoate			Preincubation with 100- γ benzoate		
	Benzoate alone	Benzoate + strepto- mycin	Inhibi- tion	Benzoate alone	Benzoate + strepto- mycin	Inhibition	Benzoate alone	Benzoate + strepto- mycin	Inhibi- tion
			per cent			per cent			per cent
0 25	9	10	0	36	28		36	34	
0 45	24	17	29	80	60	25	74	71	4
1 20	78	40	49	189	135	28	182	161	12
1 40	129	55	57	262	178	32	261	213	18
2 00	188	70	63	338	219	35	336	263	22
2 20	251	84	66	407	259	37	411	310	24
2 40	320	99	69	473	295	38	460	357	22
3 10	432	119	72	502	354	30	474	429	10

TABLE IV

Oxidation of 1.0 Mg. of Sodium m-Hydroxybenzoate by Mycobacterium lacticola Grown in Normal Medium and with m-Hydroxybenzoate As Sole Carbon Source

The cells were subjected to sonic treatment for 20 minutes. The control uptakes which were very small have been subtracted. The figures represent c.mm. of oxygen uptake. pH 6.7; temperature 37°.

Time, hrs., min.	Control		Grown in <i>m</i> -hydroxybenzoate as sole C source	
	<i>m</i> -Hydroxybenzoate	<i>m</i> -Hydroxybenzoate + 0.2 mg. streptomycin	<i>m</i> -Hydroxybenzoate	<i>m</i> -Hydroxybenzoate + 0.2 mg. streptomycin
0 30	0	0	20	17
1 00	0	0	42	38
2 00	2	0	89	70
3 00	9	0	126	106

cells. The cells grown in *m*-hydroxybenzoate are still able to oxidize it, although more slowly than the equivalent amount of living cells. A sufficient number of living cells are, however, present in both cultures so that

adaptive enzyme formation is indicated by the rate of oxidation increasing with time. Before this occurs to a significant extent, the oxidation is not inhibited by streptomycin. Thus the effects obtained with the living adapted cells are not the result of changes in permeability to the drug.

DISCUSSION

Cohen (5) has shown that streptomycin combines with nucleic acids. It seems probable that this is part of the mechanism by which the drug inhibits the formation of adaptive enzymes since nucleoproteins apparently are concerned in enzyme formation. Whether this effect of streptomycin can also explain the inhibition of growth depends on how important the formation of adaptive enzymes is to the normal functioning of the cell. We have thus far been unable to demonstrate adaptive enzyme formation in virulent *Mycobacteria* which are sensitive to streptomycin. It is probable therefore that the inhibition of adaptive enzyme formation is only one aspect of the mechanism of streptomycin action.

SUMMARY

1. Benzoic acid is oxidized in certain *Mycobacteria* by an adaptive enzyme, the formation of which is inhibited by streptomycin.

2. Some of the factors affecting the adaptive enzyme formation are described.

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THE EFFECT OF SUCCINATE ON RESPIRATION AND CERTAIN METABOLIC PROCESSES OF MAMMALIAN TISSUES AT LOW OXYGEN TENSIONS IN VITRO*

BY ROBERT F. FURCHGOTT AND EPHRAIM SHORR

(From the Department of Medicine, Cornell University Medical College, and The New York Hospital, New York)

(Received for publication, February 19, 1948)

The striking enhancement by succinate of the O_2 consumption of excised tissues suggested the possibility that this substrate might serve to maintain more normal oxidative rates in tissues exposed to the reduced oxygen tensions prevailing in circulatory shock. As part of a coordinated research program on the therapy of shock under the auspices of the Committee on Medical Research of the Office of Scientific Research and Development, this possibility was explored in a number of laboratories. Studies in this laboratory were carried out on excised tissues in order to avoid the difficulties of interpretation inherent in experiments on whole animals and to permit a more specific analysis of the respiratory and metabolic effects of succinate.

The experiments were designed with two purposes in mind. The first was to ascertain the effect of succinate on the respiration of a variety of mammalian tissues *in vitro* under the low O_2 tensions characteristic of circulatory shock. The second was to determine the influence of succinate on specific essential metabolic processes, particularly those which require oxidative energy for their maintenance. The following tissues and metabolic processes were investigated: brain (acetylcholine synthesis), liver (urea synthesis), kidney (deamination), cardiac muscle and smooth muscle ("high energy" phosphate resynthesis), and skeletal muscle.

With all of these tissues succinate in substrate concentration¹ markedly increased the O_2 consumption at low as well as at high O_2 tensions. However, in no instance was the energy resulting from the extra oxygen con-

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Cornell University Medical College.

¹ Succinate was used in these studies in concentrations which are considerably greater than those used in experiments with tissue homogenates and extracts, in which succinate is added as a catalyst to aid in oxidation of other substrates. When succinate was added in "catalytic" concentrations (about one-tenth or less of the order of concentrations used here) to the tissue preparations under investigation, no catalytic effects on respiration could be elicited except in occasional experiments with kidney slices.

sumption capable of increasing the rates of the various metabolic processes studied. Moreover, succinate competed successfully with the normal substrates of the tissues for the decreased amounts of O_2 available at low O_2 tensions, thus further reducing the rates of oxidation of those substrates. As a consequence, the amount of useful oxidative energy available for the metabolic processes studied was actually reduced under the influence of succinate, despite the over-all increase in O_2 consumption. These *in vitro* results provide no support for the concept that succinate might overcome the metabolic derangements in anoxic tissues during circulatory shock.

EXPERIMENTAL

General Methods—The tissues were obtained from dogs under sodium pentobarbital anesthesia, from cats under ether anesthesia, and from rabbits and rats after stunning. Cardiac muscle, liver, and kidney cortex were prepared as thin slices. Skeletal muscle fibers were teased from the scalenus anticus muscle of the dog by the technique of Shorr (1), and sheets of smooth muscle were stripped from dog small intestine. Suspensions of cat brain cortex and whole rat brain were made by chopping the tissue into fine particles with a razor blade. Two incubation media were employed: Ringer-phosphate solution (M/75 PO_4 , pH 7.35) with the conventional Barcroft-Warburg manometric system, and Krebs-bicarbonate solution (2) with Summerson differential manometers (3).

Succinate was added as the hexahydrated disodium salt and the concentration expressed in mg. per cent of succinic acid (44 mg. per cent of succinic acid being equivalent to 100 mg. per cent of hydrated salt). Gas mixtures containing various concentrations of O_2 and N_2 were obtained from the Ohio Chemical Company or prepared in our laboratory by means of a capillary flowmeter. The gas phase contained 5 per cent CO_2 whenever Krebs-bicarbonate solution was used. All incubations were carried out at 37.5° . Respiratory values and other metabolic data are given on the basis of wet weight. The respiratory data represent averages usually of triplicate, occasionally of duplicate or quadruplicate, determinations.

Effect of Low O_2 Tensions and Sodium Succinate on O_2 Consumption in Vitro—The effect of low O_2 tensions on oxygen consumption (QO_2) of various mammalian tissues and the influence of succinate on QO_2 of various oxygen tensions are shown in representative experiments assembled in Table I. Ringer-phosphate solution containing 100 or 200 mg. per cent of glucose was used throughout. Prior to incubation, the brain suspensions were washed in oxygenated Ringer's solution for 5 minutes. The other tissues were given a preliminary washing of from 10 to 20 minutes.

For all the tissues except brain the QO_2 in 20 per cent O_2 , in the absence of succinate, was 25 to 55 per cent lower than in 100 per cent O_2 . In the

case of brain, the QO_2 in 20 per cent O_2 was not significantly lower than in 100 per cent O_2 , probably because of the small particle size of the brain suspension. Addition of succinate, whether at high or low O_2 tensions, invariably increased the QO_2 . It is of particular interest that the QO_2 at certain reduced O_2 tensions could be raised by the addition of succinate to or above the control QO_2 in 100 per cent O_2 . Where this has occurred in the experiments in Table I, it is indicated by figures in bold-faced type.

A more detailed analysis of the effects of succinate on O_2 consumption and CO_2 production is presented in Table II. In this typical experiment the respiratory exchange of cat brain cortex under the influence of succinate was measured over the course of a 2 hour incubation in 100 per cent O_2 .

TABLE I

Effect of Low Oxygen Tensions and Sodium Succinate on Oxygen Consumption in Vitro

Incubation for 1 hour at 37.5° in Ringer- m/75 PO_4 plus 100 or 200 mg. per cent of glucose.

O_2 in gas phase	Succinic acid in medium	QO_2 (wet weight)				
		Cat brain cortex	Dog heart	Dog liver	Dog skeletal muscle	Rabbit kidney cortex
per cent	mg. per cent					
100	0	0.62	0.82	0.53	0.38	2.42
	44	0.81	1.07		0.60	
60	0		0.72			2.07
	44		0.96			3.16
20	0	0.61	0.46	0.25	0.28	1.13
	44	0.74	0.70	0.53	0.44	1.63
	88		0.86	0.73		1.73
	132		1.03		0.54	1.88
10	0	0.50				
	44	0.65				

Succinate caused an over-all increase in O_2 consumption of 19 per cent; however, there was at the same time a 12 per cent decrease in CO_2 production. Moreover, the stimulation of the rate of O_2 consumption in the presence of succinate was of a transitory character, persisting only for about 90 minutes, and the total extra O_2 consumption was even less than the theoretical amount needed to oxidize all of the succinate present to fumarate.

Table III summarizes the results of experiments similar to the experiment presented in detail in Table II. With brain, liver, heart, and skeletal muscle, it was consistently observed that (1) the stimulation of O_2 consumption by succinate at high O_2 tension was of a transitory nature, disappearing after 60 to 105 minutes of incubation; that (2) the total extra O_2 consumption

TABLE II

Effect of Succinate on O₂ Consumption and CO₂ Production of Suspension of Cat Brain Cortex

Incubation at 37.5° and 100 per cent O₂ tension in Ringer- M/75 PO₄ plus 100 mg. per cent of glucose; calculations based on wet weight of tissue; 350 mg. of tissue in 3 cc. of medium per vessel.

Time elapsed	No succinic acid			44 mg per cent of succinic acid*		
	Total O ₂ consumed	Increment in O ₂ consumption	Total CO ₂ produced	Total O ₂ consumed	Increment in O ₂ consumption	Total CO ₂ produced
min.	c mm. per mg.	c mm. per mg.	c.mm. per mg.	c.mm. per mg.	c.mm. per mg.	c.mm. per mg.
15	0.105	0.105		0.165	0.165	
30	0.230	0.125		0.320	0.155	
45	0.350	0.120		0.475	0.155	
60	0.465	0.115		0.615	0.140	
75	0.585	0.120		0.745	0.130	
90	0.695	0.110		0.870	0.125	
105	0.810	0.115		0.990	0.120	
120	0.925	0.115	0.950	1.10	0.110	0.840

* Oxidation of all succinate present to fumarate would have required 0.36 c.mm. of O₂ per mg. of tissue.

TABLE III

Effect of Succinate on O₂ Consumption and CO₂ Production at High O₂ Tension

Incubations in Ringer-M/75 PO₄ at 37.5° with and without succinate. 100 per cent O₂ in Experiments 1 to 10; 60 per cent O₂ in Experiment 11; 3 cc. of medium per vessel; succinate concentration equivalent to 44 mg. per cent of succinic acid; 100 mg. per cent of glucose in medium, except in Experiments 3, 4, and 8.

Experiment No.	Tissue	Wet weight of tissue per vessel	Length of incubation	Duration of increased O ₂ consumption with succinate	ΔO ₂ *	ΔCO ₂ †
		mg.	min.	min.	c.mm.	c.mm.
1	Brain cortex (dog)	350	90	75	+57	-11
2	Whole brain (rat)	250	90	60	+98	-2
3	Liver (rat)	250	90	75	+57	-10
4	Heart (dog)	200	120	105	+88	-6
5	" "	250	120	90	+98	
6	Skeletal muscle (dog)	330	120	105	+107	
7	Kidney cortex (dog)	150	60	>60	+141	
8	" " (rabbit)	200	60	>60	+214	
9	" " "	150	75	>75	+164	
10	" " (dog)	100	60	>60	+63	+33
11	" " "	100	60	>60	+56	+14

*"Extra" O₂ consumption in presence of succinate. 125 c.mm. of O₂ required to oxidize all succinate present to fumarate.

† Change in CO₂ production in presence of succinate

was less than would have been required to oxidize all the succinate present to fumarate; and that (3) the CO_2 production, in those experiments in which it was determined, never increased in the presence of succinate and usually was moderately depressed. With kidney cortex, on the other hand, the stimulation of O_2 consumption by succinate was well sustained throughout the periods of incubation, and, in those experiments in which the amount of tissue per vessel was sufficiently large, a greater extra O_2 consumption occurred than could be accounted for by the one-step oxidation of succinate (Experiments 7, 8, and 9, Table III). Moreover, with kidney under high O_2 tension, succinate produced an increase in CO_2 production (Experiments 10 and 11).

TABLE IV

Effect of Succinate at Low O_2 Tensions on O_2 Consumption and CO_2 Production

Incubations for 1 hour at 37.5° . Ringer- $\text{m}/75$ phosphate in Experiments 1 to 4; Krebs-bicarbonate in Experiment 5.

Experiment No.	Tissue	O_2 in gas phase	Succinic acid in medium	QO_2 (wet weight)	QCO_2 (wet weight)
		per cent	mg. per cent		
1	Liver (rat)	20	0	0.39	0.29
		20	88	0.85	0.20
2	Brain "	10	0	0.70	0.66
		10	88	0.84	0.57
3	Smooth muscle (dog)	20	0	0.16	0.18
		20	88	0.35	0.11
4	Kidney cortex "	20	0	0.88	0.66
		20	88	1.13	0.41
5	Heart (dog)	23	0	0.25	0.18
		23	88	0.77	0.10

Competition between Succinate and Normal Substrates at Reduced O_2 Tensions—At reduced O_2 tensions the increased QO_2 induced by succinate was consistently accompanied by a decreased QCO_2 . This effect is shown in Table IV, as well as in Tables VII, IX, and X. In the experiments in Table IV the decreases in QCO_2 were 31, 14, 39, 38, and 44 per cent respectively for liver, brain, smooth muscle, kidney, and heart. This effect is believed to result from the competition between succinate and normal substrates for the reduced amounts of O_2 in the tissues at low O_2 tensions. If the succinate is oxidized for the most part only as far as fumarate and competitively reduces the availability of O_2 for the oxidation of normal substrates which give rise to CO_2 , a decrease in CO_2 production would occur.

Although the extra O_2 consumption at low O_2 tensions indicates that more energy is being released in the presence of succinate than in its absence, the question arises as to whether this extra energy is utilizable for

energy-requiring metabolic processes. The experiments to be described were designed to answer this question.

Acetylcholine Formation in Brain—Fresh rat brain, after being placed in Warburg vessels with Ringer-phosphate solution containing 100 mg. per cent of glucose, was equilibrated for 15 minutes in the Warburg bath. Eserine sulfate (1:4000) was then added from the side sacs and the control vessels immediately removed for analysis. Respiration was allowed to continue for 1 or 2 hours in the incubation vessels. Extraction of acetylcholine from the tissue and medium was carried out by the method of Nachmansohn and Machado (4). The method of assay was that of Chang and Gaddum (5), in which contraction of frog rectus abdominis is used.

TABLE V

Effect of Low O₂ Tension and Added Succinate on O₂ Consumption and Acetylcholine Production of Rat Brain

Incubation at 37.5° in Ringer- m/75 PO₄ plus 100 mg. per cent of glucose.

Experiment No.	Length of incubation	O ₂ in gas phase	Succinic acid in medium	QO ₂ (wet weight)	Acetylcholine production
	hrs.	per cent	mg. per cent		γ per gm.
1	2	100		1.00	6.4
		5		0.67	5.2
		5	132	0.82	4.8
2	1	100		1.17	4.4
		5		0.77	2.9
		5	132	0.89	2.2
3	1	100		0.82	4.0
		5		0.60	2.0
		5	176	0.86	1.4
4	1	100		1.24	7.4
		5		0.67	4.4
		5	176	0.77	3.0

The results of four experiments are given in Table V. In order to obtain the desired amount of reduction in QO₂ of minced rat brain, it was necessary to reduce the O₂ tension to 5 per cent. A decrease in acetylcholine production always accompanied the decrease in QO₂ at this low tension. Although succinate increased QO₂ at the low O₂ tension by 15 to 27 per cent, it failed to increase, and actually depressed acetylcholine synthesis by 8 to 32 per cent. These results are in agreement with those of Mann *et al.* (6) as to the ineffectiveness of succinate oxidation as a source of energy for the synthesis of acetylcholine by brain tissue *in vitro* at higher O₂ tensions.

Urea Formation in Liver—Urea production in rat and dog liver slices was investigated by the method of Krebs and Henseleit (2). The incubations

were carried out in Summerson differential manometers (3) after a 15 minute equilibration period. The gas phase consisted of 5 per cent CO_2 and various percentages of O_2 and N_2 . Measurements were made of O_2 consumption, CO_2 production, and acid and urea formation. Urea was determined manometrically (2) in the same differential manometers in which respiration was measured. The values in Tables VI and VII give the amounts of urea formed during the period of incubation following the initial equilibration.

The experiments in Table VI show a marked decrease in urea production to accompany the decrease in O_2 consumption at low O_2 tensions. In Ex-

TABLE VI

Effect of Low O_2 Tension on O_2 Consumption and Urea Synthesis of Liver Slices

Incubation at 37.5° for 1 hour in Krebs-bicarbonate solution containing 75 mg. per cent of NH_4Cl ; 100 mg. per cent of glucose in Experiments 4 to 9. Gas phase, O_2 , N_2 , and 5 per cent CO_2 . Experiment 1 with dog liver, remainder with rat liver.

Experiment No.	95 per cent O_2		47 per cent O_2		20 to 23 per cent O_2^*	
	O_2 (wet weight)	Urea synthesis	O_2 (wet weight)	Urea synthesis	O_2 (wet weight)	Urea synthesis
		mg. per gm. per hr.		mg. per gm. per hr.		mg. per gm. per hr.
	0.90	0.89			0.38	0.33
	1.61	0.58			0.60	0.20
	1.01	0.53			0.41	0.18
	1.70	0.94	1.38	0.41	0.63	0.09
	1.54	0.71	0.95	0.42	0.58	0.15
	1.05	0.79	0.93	0.30	0.47	0
	1.22	0.90	0.77	0.46	0.31	0.20
	1.58	0.92	1.12	0.47	0.59	0.09
	1.20	0.93	1.13	0.34	0.68	0

* Experiments 1 to 3 with 20 per cent O_2 and Experiments 4 to 9 with 23 per cent O_2 .

periments 1, 2, 3, and 7 the decrease in urea production was roughly proportional to the decrease in O_2 . In the other experiments the percentage decrease in urea production at low O_2 tensions was much greater than the percentage decrease in O_2 . Indeed, in Experiments 6 and 9, urea synthesis was abolished in 23 per cent O_2 , whereas the O_2 decreased only about 50 per cent below that in 95 per cent O_2 . The fact that the urea synthesis sometimes decreased to a much greater extent than the O_2 consumption may indicate that the synthesis of urea is coupled with certain specific oxidations which were decreased to a greater extent than over-all O_2 consumption at low O_2 tensions.

Table VII shows the effect of succinate on the respiration and urea pro-

duction of liver slices at low O_2 tension. It is of some interest that the R.Q. in 20 per cent O_2 in the absence of succinate was always significantly higher than in 95 per cent O_2 . The addition of succinate at 20 per cent O_2 tension significantly increased the QO_2 (66, 37, and 44 per cent). However, no elevation of urea synthesis occurred, but actually a further reduction (-42, -45, and -55 per cent). Table VI also shows the concomitant decrease in CO_2 production in the presence of succinate.

Deamination in Kidney—Dog kidney slices were incubated in a Ringer-phosphate medium containing L- or DL-phenylalanine. A comparison was made of O_2 consumption and deamination, as measured by ammonia production, during 1 hour incubations in 100 and 20 per cent O_2 , with and without succinate. Total ammonia (free ammonia and amide ammonia)

TABLE VII
*Effect of Succinate on Respiration and Urea Formation of Liver Slices
at Low O_2 Tensions*

Incubation at 37.5° in Krebs-bicarbonate solution with 75 mg. per cent of NH_4Cl .

Experiment No.	Animal	O_2 in gas phase	Succinic acid in medium	QO_2 (wet weight)	$Q(O_2)$ (wet weight)	R.Q.	Urea synthesis
		per cent	mg per cent				mg. per gm per hr.
1	Dog	95	0	0.90	0.44	0.50	0.89
		20	0	0.38	0.27	0.71	0.33
		20	88	0.63	0.22	0.35	0.19
2	Rat	95	0	1.61	1.10	0.68	0.58
		20	0	0.60	0.43	0.72	0.20
		20	44	0.82	0.28	0.34	0.11
3	"	95	0	1.01	0.44	0.44	0.53
		20	0	0.41	0.32	0.78	0.18
		20	44	0.59	0.10	0.17	0.08

was determined on samples before and after incubation as follows: The tissue and medium from a Warburg vessel were mixed with 10 cc. of 5 per cent trichloroacetic acid. To 5 cc. of the trichloroacetic acid filtrate was added 1 cc. of 10 per cent H_2SO_4 , and the solution heated for 10 minutes in a boiling water bath to hydrolyze any amides. The ammonia was then collected by the method of Conway and Byrne (7), and determined colorimetrically with Nessler's reagent (8).

In all three experiments in Table VIII, a significant fall in deamination accompanied the fall in QO_2 when O_2 tension was reduced to 20 per cent. Addition of succinate at the low O_2 tension increased QO_2 by 79, 28, and 10 per cent in Experiments 1, 2, and 3 respectively. These increases were accompanied in Experiments 1 and 2 by a further decrease (-23 per cent

and -22 per cent) in the rate of deamination; in Experiment 3, in which the smallest increase in QO_2 occurred with succinate, there was no significant change in the amount of deamination. The decrease in deamination with added succinate in the first two experiments is probably due to a competition for O_2 between the deamination enzyme systems and the succinic oxidase system.

Phosphorylation in Cardiac Muscle—Phosphate transfer in slices of dog cardiac muscle was studied at 100 and 20 per cent O_2 tension by methods previously developed in this laboratory (9). The slices were first equilibrated for 20 minutes at 37.5° in a Ringer-phosphate medium. Radio-

TABLE VIII

Effect of Low Oxygen Tension and Succinate on Ammonia Formation of Dog Kidney Slices

Incubation at 37.5° for 1 hour in Ringer- $M/75 PO_4$ plus 0.01 M phenylalanine.

Experiment No.	O_2 in gas phase	Succinic acid in medium	QO_2 (wet weight)	Ammonia production*
	per cent	mg per cent		γN per gm. per hr
1†	100		1.56	205
	20		0.77	102
	20	88	1.38	79
2‡	100		1.70	315
	20		0.88	184
	20	88	1.13	143
3‡	100		2.17	378
	20		1.15	142
	20	88	1.26	141

* Ammonia N also includes ammonia converted to amide during the period of incubation.

† L-Phenylalanine present.

‡ DL-Phenylalanine present.

active phosphorus was added as inorganic phosphate and the incubation continued for 10 minutes. The tissues were then chilled and extracellular inorganic phosphate was separated by the washing technique previously described. Analyses were made of the concentrations of inorganic phosphate, labile phosphate of adenylyl pyrophosphate (APP), and creatine phosphate (CP), as well as of the radioactivity of each fraction. Respiratory measurements were carried out in Warburg vessels simultaneously with the incubation with radioactive phosphate.

The results of two experiments are summarized in Table IX. The QO_2 of cardiac muscle was reduced by 48 and 42 per cent in these experiments when O_2 tension was reduced from 100 to 20 per cent. Addition of 88 mg.

per cent of succinate at the lower tension increased the QO_2 above that found in 100 per cent O_2 . The stimulation of O_2 consumption by succinate was accompanied by a significant decrease in CO_2 production in the experiment in which this was measured. At 20 per cent O_2 there was a considerable reduction in the concentration of CP and the labile phosphate of the APP fraction. The addition of succinate at 20 per cent O_2 led to a further significant reduction in the concentration of both CP and APP, despite the marked enhancement of O_2 consumption.

The changes in concentration of CP and the labile phosphate of APP are indicative of a reduction in the rate of synthesis of these high energy organic phosphates at 20 per cent as compared with 100 per cent O_2 , and a still

TABLE IX

Effect of Low Oxygen Tension and Succinate on Phosphate Concentrations and Radioactive Phosphate Transfer in Slices of Dog Cardiac Muscle

Incubation at 37.5° in Ringer- M/75 PO_4 . Incubation with radioactive PO_4 for 10 minutes. Incubation for respiratory data for 1 hour. All specific activities given relative to the specific activity of the intracellular inorganic phosphate taken as 100.

O_2 in gas phase	Succinic acid in medium	Respiration		Creatine phosphate		Labile phosphate of adenyl pyrophosphate	
		QO_2 (wet weight)	QCO_2 (wet weight)	Concentra- tion	Relative specific activity	Concentra- tion	Relative specific activity
per cent	mg. per cent			mg. per cent P		mg. per cent P	
100	0	0.69	0.60	15.7	48.6	12.7	21.2
20	0	0.36	0.30	6.4	55.6	5.9	30.2
20	88	0.70	0.19	2.2	45.5	4.2	32.1
100	0	0.76		28.3	52.2	10.9	35.2
20	0	0.44		12.6	42.6	9.0	19.6
20	88	1.00		3.3	83.0	7.0	23.3

further reduction in the presence of succinate at 20 per cent O_2 . The radioactive phosphate determinations bear out this inference. In interpreting the radioactivity data it is necessary to consider not only the specific activities but also the total activities of the phosphate fractions into which radioactive phosphate was introduced. For instance, in the second experiment the relative specific activities of the CP and labile phosphate of APP were greater with succinate than in its absence. However, the relative *total* activities (relative total activity = concentration $\times$ relative specific activity) of these high energy phosphates in 20 per cent O_2 were decidedly lower in the presence of succinate. On this basis of relative total activity, the data in Table VIII qualitatively indicate a slower rate of synthesis of high energy phosphates at 20 per cent than at 100 per cent O_2 , and an even

slower rate of synthesis at 20 per cent O_2 in the presence of succinate than in its absence.

The values given in Table IX for the specific activity of the intracellular inorganic phosphate relative to those of CP and APP are probably somewhat lower than the true values for the following reasons. After a 10 minute incubation the specific radioactivities of CP and the labile phosphate of APP are lower than that of the intracellular inorganic phosphate. During the washing at 2° to remove the extracellular inorganic phosphate, there is some hydrolysis of these organic fractions to inorganic phosphate (9), which serves to dilute the specific activity of the original intracellular inorganic phosphate. A similar situation for *in vivo* experiments with radioactive phosphate has been discussed by Kalckar *et al.* (10).

TABLE X

Effect of Low O_2 Tension and Succinate on Respiration and Concentration of Creatine Phosphate and Adenyl Pyrophosphate of Intestinal Smooth Muscle

Incubation at 37.5° for 1 hour in Ringer- $m/75 PO_4$ plus 200 mg. per cent of glucose.

O_2 in gas phase	Succinic acid in medium	QO_2 (wet weight)	QCO_2 (wet weight)	Creatine phosphate	Labile phosphate of adenyl pyrophosphate
<i>per cent</i>	<i>mg. per cent</i>			<i>mg. per cent P</i>	<i>mg. per cent P</i>
100	0	0.25	0.25	1.92	3.38
20	0	0.13	0.14	1.52	3.31
20	88	0.22	0.11	0.78	2.34
100	0	0.32		2.14	6.94
20	0	0.13		1.17	4.64
20	88	0.49		0.86	3.00

The values for the specific activity of creatine phosphate in 20 per cent O_2 in the presence of succinate are likely to be less accurate than the other values in Table IX. This is a consequence of the low concentration of that fraction and the correction for the precipitation of about 1 per cent inorganic phosphate along with creatine phosphate in the method used (9).

Phosphorylation in Intestinal Smooth Muscle—The two experiments reported in Table X show the effect of low O_2 tension and succinate on the respiration and concentrations of the creatine phosphate and labile phosphate of adenyl pyrophosphate of dog intestinal smooth muscle during a 1 hour incubation. The reduction of O_2 tension from 100 to 20 per cent decreased O_2 consumption by 48 and 69 per cent in the two experiments, and also caused significant reductions in the concentrations of CP and labile phosphate of the APP. Addition of sodium succinate at the lower O_2 tension increased O_2 consumption markedly. However, as in the case of cardiac muscle, succinate caused still further decreases in the concentrations of CP

and the labile phosphate of APP of the smooth muscle. The inability of succinate oxidation to increase high energy phosphate resynthesis in dog intestinal smooth muscle is in accord with the previous report from our laboratory (11) that succinate does not serve as an energy-yielding substrate for the maintenance of rhythmic contractions of rabbit intestinal smooth muscle.

DISCUSSION

The experiments reported here demonstrate that succinate is indeed capable of increasing the oxygen consumption of a variety of tissues *in vitro* at low oxygen tensions. However, it is very evident that the energy provided by the oxidation of succinate cannot be used for the maintenance of any of the important energy-requiring metabolic processes which we have studied (acetylcholine synthesis in brain, urea synthesis in liver, and phosphorylations in cardiac muscle and intestinal smooth muscle). Indeed, these metabolic processes are actually decreased in the presence of succinate below the control values at low O_2 tensions. On the basis of these *in vitro* experiments it would not seem likely that succinate would exert a corrective influence in circulatory shock, since its oxidation would not increase, and might possibly decrease, the rates of energy-requiring metabolic processes in tissues receiving inadequate supplies of O_2 . The apparent usefulness of sodium succinate in the treatment of experimental shock in animals (12, 13) has recently been ascribed to the alkalinizing effect of the sodium ion rather than to a metabolic effect of the succinate ion, since sodium bicarbonate proved equally effective (14).

In addition to the relevance of these *in vitro* experiments to succinate therapy in circulatory shock, they are also pertinent to a consideration of the rôle of succinate in intermediary metabolism. The first point of interest is the extent to which succinate in substrate quantities is oxidized in the several tissues studied. In an earlier investigation Quastel and Wheatley (15, 16) showed by chemical analysis that the end-products of succinate oxidation by minced brain and skeletal muscle were fumarate and malate. More recently Elliott and coworkers (17, 18) concluded on the basis of respiratory and other data that brain slices, liver slices, and testes *in vitro* oxidized succinate for the most part only as far as fumarate, but that kidney cortex slices oxidized succinate in large part to CO_2 and H_2O . The respiratory data of the present study are in agreement with the results of Quastel and Wheatley and Elliott and coworkers. In the case of all the tissues investigated, except kidney, the respiratory data at high oxygen tensions indicate that very little, if any, of the added succinate was oxidized beyond fumarate.

Another observation of interest is furnished by the regularity with which

stimulation of O_2 consumption by succinate at low O_2 tensions was accompanied by a depression of CO_2 production. This we have ascribed to a successful competition by succinate for the reduced amounts of oxygen available for tissue oxidations at low oxygen tensions, with a resulting reduction in the extent to which normal substrates are oxidized. The evidence for this is indirect, and is based on the assumption that the same mixture of foodstuffs is being oxidized in the presence and absence of succinate, thus yielding the same r.q. under both circumstances. More direct evidence for such a competition is provided by the observations on the reduced deamination of phenylalanine by kidney at low O_2 tensions in the presence of succinate. This deamination requires O_2 ; hence its reduction suggests the reduced availability of O_2 in the presence of succinate.

Further evidence that the reduction of CO_2 at low tensions in the presence of succinate is due to the reduction in the amount of normal substrates being oxidized is furnished by the experiments in which the rates of various energy-requiring metabolic processes were depressed by the addition of succinate at low O_2 tensions. The validity of this evidence is dependent upon the assumption that the rates of energy-requiring metabolic processes are dependent on the rates of oxidation of normal substrates.

It is of particular interest that these depressions in energy-requiring metabolic processes occurred despite significant increases in over-all oxidative energy resulting from the addition of succinate. It is suggested that the portion of the oxidative energy contributed by added succinate differs from that furnished by normal substrate oxidation, in that it is either totally or almost completely unavailable for "useful" work. The one situation in which this inference can be dealt with in semiquantitative fashion is found in the experiments on phosphorylation in cardiac and smooth muscle. These experiments may be considered in the light of the available evidence as to the coupling of high energy phosphate formation with the oxidation of succinate to fumarate.

Experiments with tissue extracts by Belitzer and Tsibakowa (19), Ochoa (20), Colowick *et al.* (21, 22), and Potter (23) indicate that such a coupling may occur. The results of Ochoa suggest that there may be one high energy phosphate group formed for each molecule of succinate oxidized. However, from the data in Tables IX and X, it is apparent that, if such coupling takes place in intact cardiac and smooth muscle, the number of phosphorylations per oxidation is much below the 1:1 ratio postulated. For example, in the first experiment with smooth muscle in Table X, the decrease in the O_2 consumption of normal substrates in the presence of succinate at 20 per cent O_2 tension can be estimated to be about 0.03 cc. per gm. per hour on the basis of the decrease in QCO_2 (assuming that no CO_2 is liberated by the succinate oxidation). At the same time, the increase in

O₂ consumption due to the oxidation of added succinate may be estimated to be about 0.12 cc. per gm. per hour (QO_2 with succinate minus QO_2 without succinate + 0.03). On the basis of a 1:1 ratio for phosphorylation coupled with oxidation of succinate and a 3:1 ratio of phosphorylation per oxygen atom used in oxidizing normal substrates (19, 24), an increase in the rate of phosphorylation would have been predicted. Actually, the creatine phosphate and adenylyl pyrophosphate concentrations indicate that the rate of phosphorylation was markedly decreased in the presence of succinate.

The experiments on phosphorylation in cardiac muscle with radioactive phosphate are also of interest with respect to the manner in which inorganic phosphate passes from the extracellular into the intracellular phase. In a previous paper from this laboratory (9) it was reported that in cardiac muscle slices incubated at 37.5° for 30 minutes or more with radioactive phosphate, the specific activities of the phosphate of creatine phosphate and of the terminal phosphate of adenosine triphosphate attained the same level as the specific activity of the intracellular inorganic phosphate. In the present investigation, the reduction of the incubation period to 10 minutes resulted in decidedly higher specific activity for the intracellular inorganic phosphate than for creatine phosphate and the labile phosphate of adenylyl pyrophosphate. Apparently the incubation time was too short for the phosphorylation cycle to bring the specific activity of the creatine phosphate and labile phosphate of the adenylyl pyrophosphate to the specific activity level of the intracellular inorganic phosphate. This differential in specific activities is further evidence against the concept that inorganic phosphate is transported across the cell boundary by first being incorporated into creatine phosphate or adenylyl pyrophosphate.

We wish to acknowledge the technical assistance of Mathilda Fischl Goldsmith.

SUMMARY

1. Significant depressions in the rates of O₂ consumption of viable preparations of various mammalian tissues *in vitro* occurred under conditions of low O₂ tension. The depressions in rates of O₂ consumption were accompanied by corresponding depressions in the rates of a variety of metabolic processes, such as urea synthesis in liver slices, acetylcholine synthesis in brain suspension, deamination in kidney slices, and phosphorylation in cardiac muscle slices and smooth muscle strips.

2. An exploration was made of the possible usefulness of sodium succinate in overcoming these depressions in metabolic rates under conditions of low O₂ tension.

3. Succinate, in substrate quantities (44 mg. per cent or more in terms of succinic acid), was capable of markedly increasing the rates of O_2 consumption of all the tissues studied here, at reduced O_2 tensions as well as in 100 per cent O_2 .

4. The energy from extra oxygen consumption in the presence of succinate, however, was found to be incapable of increasing the rates of metabolic processes depressed under conditions of low O_2 tension. Indeed, in the presence of an increased O_2 consumption produced by succinate, the rates of the metabolic processes studied were even further depressed.

5. The respiratory data have been interpreted as indicating that in these experiments succinate was oxidized for the most part only to fumarate. At low O_2 tensions, the succinate competed with normal substrates for the limited concentration of O_2 present, and thus further depressed the rates of oxidation of normal substrates. As a result, the rates of metabolic processes, dependent for energy on the oxidation of normal substrates, were further depressed in the presence of succinate at low O_2 tensions.

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A SYNTHESIS OF ALLOCHOLESTEROL AND EPIALLOCHOLESTEROL*

By HERBERT McKENNIS, JR., AND GEORGE W. GAFFNEY

(From the Department of Physiological Chemistry, The Johns Hopkins University,
School of Medicine, Baltimore)

(Received for publication, April 14, 1948)

By application of a Meerwein-Ponndorf reduction, Schoenheimer and Evans (1, 2) obtained from 3- Δ^4 -cholestenone a molecular compound of allocholesterol and epiallocholesterol. These workers separated these diastereoisomers by use of digitonin and demonstrated that the "allocholesterol" of Windaus (3) was in fact a molecular compound of allocholesterol and cholesterol. Later Eck and Hollingsworth (4) showed that three isomeric cholestadienes, $\Delta^{2,4}$, $\Delta^{3,5}$, $\Delta^{4,6}$, upon treatment with hydrogen chloride and subsequent contact with aluminum oxide, yielded the allo-epiallocholesterol molecular compound. The low yields in this latter synthesis, however, do not at present lead to general use of this interesting reaction for the preparation of allocholesterol.

Although Stoll (5) reported that allocholesterol occurs naturally and can be separated from cholesterol by virtue of the insolubility in ether of a complex between pyridine and allocholesteryl *p*-toluenesulfonate, he was able to obtain only traces of the substance from bile stones, liver, brain, and egg yolk. The results of other workers (6-8) show quite conclusively that allocholesterol and epiallocholesterol can only be present in very low concentration. This militates against the isolation of any but small amounts from customary natural sources.

Recently Nystrom and Brown (9) have reported a very elegant method for reducing carbonyl and other —C=O groups to the corresponding alcohol by means of lithium aluminum hydride (10-12). Although their paper (9) stated that carbon to carbon double bonds in certain unsaturated ketones are reduced by this reagent, it seemed to us that 3- Δ^4 -cholestenone would, when properly treated with lithium aluminum hydride, yield allocholesterol and epiallocholesterol.

It was found in our study that the product from the reaction of cholestenone and lithium aluminum hydride in absolute ether is composed of allocholesterol and epiallocholesterol. This mixture, which was characterized by a quantitative conversion to $\Delta^{3,5}$ -cholestadiene (1), was obtained even when the ketone was treated with lithium aluminum hydride in 5-fold excess over that amount calculated to effect reduction of the

* Aided by a grant from the John and Mary R. Markle Foundation.

nuclear double bond. Upon treatment with digitonin the mixture afforded allocholesterol and epiallocholesterol in approximately equal amounts and would, therefore, appear to consist of equal parts of the two expected diastereoisomers. The yield of mixed isomers was quantitative. Since the amount of mixed isomers reported for the Meerwein-Ponndorf reduction of the ketone is 52 per cent of the calculated amount, the present method appears to be superior in regard to yield as well as to convenience and economy of time.

EXPERIMENTAL

Reduction of Cholestenone with Lithium Aluminum Hydride—A solution of 0.500 gm. of cholestenone in 40 cc. of absolute ether was dropped slowly (20 minutes) with agitation into an excess of lithium aluminum hydride in 100 cc. of absolute ether. There was a slight evolution of heat, and the ether refluxed perceptibly into the condenser which was protected from the atmosphere by a soda lime tube. The excess of lithium aluminum hydride was decomposed by the addition of acetone, which obviated the production of nascent hydrogen. An additional 100 cc. of ether served to keep the alcoholates in suspension. The mixture was then treated with 100 cc. of 3 N NaOH, and the ether solution was washed several times with fresh portions of base. After drying over anhydrous sodium sulfate, the ether solution was evaporated to dryness under diminished pressure at room temperature. The residue of mixed epimers, which weighed 0.500 gm., melted over a range in the vicinity of 100°.

Cholestadiene from Reduction Product—100 mg. of mixed epimers in 30 cc. of 95 per cent alcohol were filtered to remove a trace of insoluble material. 4 drops of concentrated hydrochloric acid were added. The mixture was refluxed for 4 hours. It was then cooled and diluted cautiously with water until precipitation was completed. The product was collected after the mixture had remained overnight in the refrigerator. The yield of colorless needles, m.p. 79°, was 95 mg.; $[\alpha]_D^{25} = -104^\circ$; $c = 2.00$ in benzene. The specific rotation and melting point are in agreement with the recorded values (1) for $\Delta^{3,5}$ -cholestadiene.¹ For analysis² a sample was recrystallized several times from ethanol.

$C_{27}H_{46}$ (368.6).	Calculated.	C 87.96, H 12.04
	Found	" 87.81, " 12.14
		" 87.75, " 12.07

¹ Although the compound obtained by treating allocholesterol and epiallocholesterol with dilute alcoholic HCl was originally described (1) as $\Delta^{3,4}$ -cholestadiene, the available evidence (1, 13, 14) indicates that the product is $\Delta^{3,5}$ -cholestadiene.

² Microanalyses by Dr. G. Oppenheimer, California Institute of Technology.

Molecular Compound of Allocholesterol and Epiallocholesterol from Reduction Product—A sample of reduction product was recrystallized several times from acetone and finally from acetone-ether. The colorless needles melted at 141° . The recorded values are 141° (1) and $140\text{--}141^{\circ}$ (4); $[\alpha]_D^{24} = +86.3^{\circ}$; $c = 1.26$ in carbon tetrachloride. Eck and Hollingsworth (4) reported $[\alpha]_D^{25} = +85.6^{\circ}$; $c = 1.25$ in carbon tetrachloride.

Isolation of Allocholesterol—250 mg. of mixed epimers were dissolved in 25 cc. of absolute ethanol. To this solution was added a solution³ prepared by adding 40 cc. of absolute ethanol to a slurry of 500 mg. of digitonin and 10 cc. of distilled water. A precipitate formed immediately. The mixture was allowed to stand overnight and then was filtered. The collected digitonide was washed with absolute ether and then extracted for 20 hours with absolute ether. The digitonide was then allowed to dissolve in 15 cc. of dry pyridine. The clear pyridine solution was treated with 100 ml. of absolute ether. The precipitated digitonin was collected by centrifugation, filtration being impossible, and then washed with additional quantities of ether. The combined mother and wash liquors from the digitonin were then concentrated to dryness *in vacuo* at room temperature. The residue of allocholesterol melted at $131\text{--}132^{\circ}$, in agreement with recorded values (1, 4); $[\alpha]_D^{25} = +44^{\circ}$; $c = 1.08$ in benzene. The yield was 110 mg., or 44 per cent of the weight of mixed epimers. Recrystallization from alcohol-water served to lower the melting point by several degrees. In this connection, it is interesting to note that Schoenheimer and Evans (1) suggested that the compound is easily solvated and reported that it gave unsatisfactory analytical figures on combustion.

Allocholesteryl Acetate—A sample of the allocholesterol after treatment with pyridine and acetic anhydride (1) yielded crystals melting at 85° , in agreement with the recorded value for the acetate.

Isolation of Epiallocholesterol—The combined mother and wash liquors from the preparation of allocholesterol digitonide were concentrated to dryness under diminished pressure at $30\text{--}35^{\circ}$ (1). The crystalline residue was dissolved in the ether solution obtained from the extraction of the digitonide. The solution was filtered through a pledget of cotton to remove a trace of colloidal digitonin. Upon evaporation of the ether solution 110 mg. of crude epiallocholesterol, m.p. $80\text{--}82^{\circ}$, were obtained. The product at this point tends to be oily (1) unless carefully purified cholestene is used in the reduction.

$\Delta^{3,5}$ -Cholestadiene from Allocholesterol and from Epiallocholesterol—

³ The presence of a few undissolved particles does not interfere with the separation. The procedure is essentially that of Schoenheimer and Evans (1), with some modification apparently made necessary by the uncertain composition of commercial digitonin.

100 mg. of epiallocholesterol (above) were treated with dilute alcoholic HCl according to the general procedure. The yield of $\Delta^{3,5}$ -cholestadiene, m.p. 79°, was 94 mg. The melting point was not depressed by admixture with authentic material. The allocholesterol yielded cholestadiene in quantitative yield under the same conditions.

SUMMARY

From the reduction of cholestenone with lithium aluminum hydride in absolute ether there was obtained a quantitative yield of a mixture containing allocholesterol and epiallocholesterol in approximately equal amounts.

The authors are grateful to Dr. Arthur St. André and Dr. C. R. Scholz, Ciba Pharmaceutical Products, Inc., for supplies of cholestenone.

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THE DEPOSITION OF TRIENOIC FATTY ACIDS IN THE FATS OF THE PIG AND THE RAT*

By B. W. BEADLE, O. H. M. WILDER, AND H. R. KRAYBILL

(From the American Meat Institute Foundation, University of Chicago, Chicago)

(Received for publication, March 17, 1948)

It has long been known that the composition of the depot fat of an animal may be altered markedly by the food fat. According to Burr and Barnes (1), the appearance of dietary fat in milk was observed by Stohmann in 1869 (2) and in body fat by Lebedeff in 1882 (3). Detailed studies of these effects were made by a number of workers, notable among whom are Ellis and coworkers (4-7), Jackson (8), Shorland and De la Mare (9), and Brown and coworkers (10-14). Much of this work was done on the hog, because of the economic disadvantages of soft bacon and other soft pork, but a number of workers (10-16) pointed out that the body lipides of the pig, rat, and chicken are changed profoundly by the diet. Anderson and Mendel (15) reported that rats tend to deposit fats in much the same manner as do fattening hogs, but Brown (13) found evidence for believing that polyunsaturated fatty acids are more readily deposited by the rat than by the pig. Ellis and coworkers (4-7), in studies on soft pork, found that the amount of linoleic acid in the lard was altered markedly by the amount of linoleic acid in the diet. For example, pig fat laid down from a carbohydrate diet contained 1.9 per cent, while that laid down from a soy bean diet contained 30.6 per cent of linoleic acid. Much the same sort of data can be found in the literature for various fatty acids. A notable exception is linolenic acid. Ellis and Isbell (4) expressed surprise at finding only very small amounts of linolenic acid in the fat of hogs fed soy beans and at the failure to find it in all samples in spite of the fairly large amounts of soy beans fed. They stated that, "The linolenic acid because of its high degree of unsaturation was evidently either metabolized or converted to a more saturated acid and then deposited." The amounts found were 0.02 and 0.50 per cent. Hilditch and Shorland (17) found small amounts of linolenic acid in sheep liver fat from pasture-fed animals. Bloor has stated in a recent monograph (18), that linolenic acid is a marked example of selection in the use of fat for energy production because this acid has been shown to be almost completely eliminated from the food fat before storage. Brown and coworkers (10-14) suggested that when the experimental diets

* Presented before the Chicago Section of the American Chemical Society and Section C of the American Association for the Advancement of Science at Chicago, December 26, 1947.

contained either arachidic or highly unsaturated C_{20} and C_{22} acids the unsaturated acids appeared in the depot fats of pigs and rats.

In the present paper we shall show that three double bond fatty acids may be stored in pig fats in very large amounts, as much as 11.4 per cent having been found in the fat from certain "yellow fat" pigs. The feeding of flaxseed to rats in an amount sufficient to provide about 15 per cent linseed oil in the ration resulted in the deposition of trienoic acids in amounts as great as 28 per cent of the depot fats.

EXPERIMENTAL

Yellow Fat from Hogs—Three samples of lard were obtained from a commercial meat packing house in regular operation. One of these samples was ordinary lard rendered from mixed killing and cutting fats, and the other two samples were rendered from the fat of "yellow" carcasses which had been rejected by the authorized inspector. Table I shows the color of these three samples as expressed in Lovibond units. It is evident that

TABLE I
Color Evaluation of Samples of Swine Fat

Sample No.	Lard	Color, Lovibond units	
		Yellow	Red
1	Ordinary	5	0.4
2	Yellow	6	0.8
3	"	35	5.9

while there was little difference between Samples 1 and 2, Sample 3 was extremely dark yellow, with a higher proportion of red than was present in the control.

The samples were analyzed for the types of fatty acids present by the spectrophotometric method developed by Mitchell *et al.* (19) and by employing the constants published by Beadle and Kraybill (20). Fig. 1 shows the absorption spectra of the alkali-isomerized soaps in ethanol. The absorption in the region of 2680 Å is typical of a conjugated trienoic fatty acid. No significant differences are noted in the region above 3200 Å. Table II shows the analytical data on these samples. The iodine numbers were obtained by the Wijs method. The iodine values of the yellow lards were 27 to 30 units higher, and the trienoic acid contents, calculated as linolenic acid, were approximately 10 times higher than those of the control lard. The fatty acid composition of the control was typical for lard, based on the analysis of several hundred samples in our laboratories.

The yellow color in these fats could not be extracted along with the un-

saponifiable matter. The color was therefore not due to carotenoid pigments of the type found in grains and grasses.

Bromination in anhydrous ethyl ether of the unsaturated acids obtained from the yellow lard resulted in the precipitation of polybromides which melted at 167–168.5°. Recrystallization from benzene yielded crystals with a melting point of 173.0–173.3°. The addition of about 10 per cent

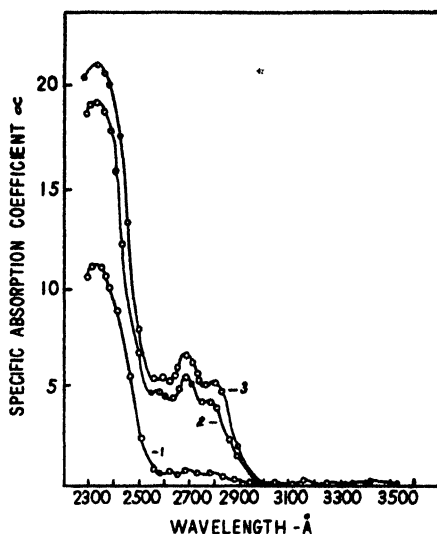


FIG. 1. The absorption spectra of the alkali-isomerized soaps from lard (with ethanol as the solvent). Curve 1, control lard; Curves 2 and 3, "yellow fat" lard.

TABLE II
Spectrophotometric Analysis of Swine Fat (on Basis of Mixed Acids)

Sample No.	Lard	Iodine value	Arachidonic	Linolenic	Linoleic	Oleic	Saturated
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
1	Ordinary	71.9	0.50	0.94	11.9	51.2	35.46
2	Yellow	101.5	0.77	11.4	16.0	43.0	28.83
3	"	98.7	1.22	9.0	15.3	47.0	27.48

of known hexabromostearic acid prepared from linseed oil (m.p. 180.5–180.7°) did not change the melting point except to raise it a few tenths of a degree. Likewise, the addition of about 10 per cent of these crystals to the known hexabromostearic acid did not lower the melting point of the known crystals more than a few tenths of a degree.

Feeding of Flaxseed to Rats—Twelve young male albino rats, weighing from 64 to 100 gm., were divided into two groups of six each at random and

confined in wire-floored cages in a constant temperature room at 24.4°. Food and water were supplied *ad libitum*. The rations which were fed to the respective groups are shown in Table III. The animals were weighed each week. One rat in Group 2, on the flaxseed ration, died during the 2nd week, leaving only five in that group. One animal in each group was killed

TABLE III
Rations Used in Feeding of Rats

	Group 1, control	Group 2, flaxseed
	<i>per cent</i>	<i>per cent</i>
Ground yellow corn.	44.7	37.2
Soy bean oil meal	30.0	10.5
Dried skim milk	5.0	5.0
Dehydrated alfalfa meal	4.0	4.0
Dried brewers' yeast	2.0	2.0
Corn oil	13.0	
Vitamin A (3000) and D (400) oil	0.3	0.3
Pulverized limestone	0.5	0.5
Iodized salt	0.5	0.5
Ground flaxseed		40.0

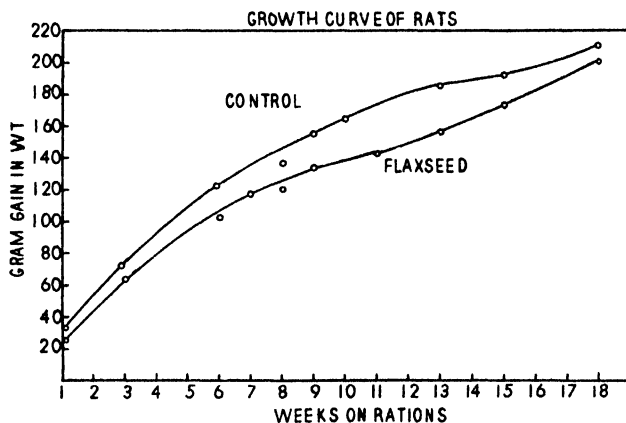


FIG. 2. Weight gain data for rats. Curve 1, control group; Curve 2, group fed flaxseed diet.

during the 19th week for fat analysis, and the remaining animals were killed during the 21st week. The average weight gains for each group of rats are shown by the curves in Fig. 2. The basal ration had been used previously as a stock ration, but was modified for the control group to include corn oil in an amount approximately equivalent to the oil received by

the rats in Group 2 on the flaxseed diet. The amount of linseed oil in the flaxseed used was 36.61 per cent, as determined by the official method of the Association of Official Agricultural Chemists. The linseed oil content was thus about 14.5 per cent of the ration. The content of linolenic acid in the oil was 45.78 per cent as determined by the spectrophotometric method, and the linolenic acid content of the flaxseed ration was 6.6 per cent.

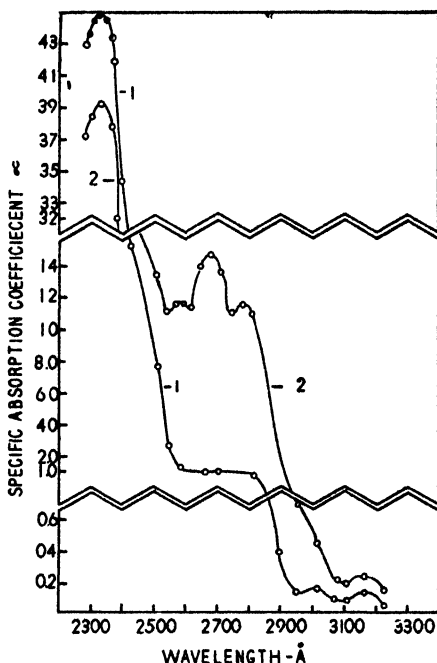


FIG. 3. Absorption spectra of alkali-isomerized soaps from rat fat (with ethanol as solvent). Curve 1, control rat; Curve 2, rat fed on flaxseed diet.

At the end of the indicated feeding periods, the depot fats were obtained from the rats in the following manner: The rats were killed in a lethal chamber by the use of chloroform and the carcasses chilled in a refrigerator at 4° for a few hours. Immediately following this chilling period, the animals were skinned and the depot fatty tissue trimmed from the carcasses. The depot tissue samples were placed in a vacuum oven for 30 hours at 50° in order to remove excess water and then extracted in a Soxhlet extractor by the use of petroleum ether during a period of 16 hours. The petroleum ether was removed under reduced pressure with some warming in a water bath, and the resulting fat subjected to the same analytical procedures as

were described for the fat from swine. Fig. 3 shows the ultraviolet absorption spectra of the alkali-isomerized soaps in ethanol. The strong similarity of these curves to those obtained from the lard samples is at once evident. The intense absorption exhibited at 2340 Å by the fat from the control animal is due to the presence of a large amount of linoleic acid. The region at 2680 Å, showing the presence of trienoic acids, indicates that the flaxseed-fed rat deposited a great deal more of this material than did the control rat. Table IV presents the analytical data obtained on the individual rats throughout Groups 1 and 2. The samples of fat obtained from the flaxseed-fed rats were not noticeably darker yellow than those from the controls.

TABLE IV
Spectrophotometric Analysis of Rat Depot Fat (On Basis of Mixed Acids)

Diet	Animal No.	Iodine value	Arachidonic	Linolenic	Linoleic	Oleic	Saturated
			<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Control	800	127.49	1.10	1.30	53.42	25.90	18.28
"	801	124.12	0.89	1.23	52.49	25.05	20.34
"	802	124.08	1.13	1.26	52.02	24.98	20.61
"	803	127.76	1.48	1.31	54.06	23.49	19.66
"	804	126.21	0.79	1.16	53.15	26.58	18.32
"	805	126.44	0.82	1.13	54.03	25.09	18.93
Average		126.02	1.04	1.23	53.20	25.18	19.36
Flaxseed-fed	807	150.06	1.42	25.33	28.21	27.54	17.51
"	808	158.00	1.22	27.59	27.91	30.86	12.42
"	809	151.53	1.57	24.73	29.49	27.89	16.32
"	810	155.29	1.28	26.42	29.85	27.26	15.19
"	811	152.56	1.40	25.93	28.37	28.25	16.05
Average		153.49	1.38	26.00	28.77	28.36	15.50

Bromination of the unsaturated fatty acids from the flaxseed-fed rats in anhydrous ethyl ether resulted in the precipitation of polybromides which melted at 169–170°. After two recrystallizations from benzene the melting point was 176.0–176.2°. The addition of 10 per cent of known hexabromostearic acids did not change the melting point, except to produce an apparent rise of 0.2°, which would be explained as a result of dilution of the impurities in the 176° sample. Likewise, the addition of 10 per cent of the crystals obtained from the rat fat to the known hexabromostearic acid did not change the melting point of the hexabromostearic acid. Furthermore, when the polybromides from the yellow lard were added to the polybromides obtained from the flaxseed-fed rats, there was no change in the melting point.

DISCUSSION

A search of the literature has not brought to light any report of hog fat containing more than minor amounts of linolenic acid. Brown and co-workers (10-14) reported about 2.7 per cent of highly unsaturated acids in the fat of pigs which had been fed a diet containing 14 per cent of fish oil, and that the iodine number of this fat was about 10 units higher than that of the control. They pointed out that rat fat is more easily affected by changes in the diet than is pig fat, for they noted much greater changes in the characteristics and composition in the fat of the rat on the fish oil diet. In experiments on the feeding of fish oils to rats, they found that an approximate equilibrium was reached in 4 to 6 weeks, and that the stored fat contained about the same amount of highly unsaturated acids as was present in the diet (10). Their conclusions were broadly the same as ours regarding the rat, although we have found that the rat deposited about 4 times as much linolenic acid as was present in the diet. While both Brown and Ellis obtained experimental evidence that highly unsaturated fatty acids may be deposited in the fat of the pig, the amounts found were small, and the general impression seems to have remained that the animal tends to metabolize or otherwise alter the fatty acids with unsaturation greater than two double bonds before depositing them in the stored fat.

Our data, based on spectrophotometric analyses as well as on bromination data, show that pig fat can, under certain conditions, contain up to 11.4 per cent of trienoic acids calculated as linolenic acid. This situation occurs only rarely, about 51 such animals having been encountered by one large packer in the course of a year. In attempting to explain the possible causes for this fat with the high content of polyunsaturated acids, we were naturally led to suspect an unusual diet, in view of the known literature dealing with the deposition of dietary fat. Substances in the diet responsible for the deposition of such large amounts of trienoic acid would necessarily be substances containing large amounts of such components. Because we found no evidence of unusually large amounts of acids containing more than three double bonds (other than the expected amounts of arachidonic acid), it did not seem likely that the effect could be attributed to fish oils. The oils would then logically be suspected as being vegetable in origin. Such oils as peanut, cottonseed, and corn do not contain more than minor quantities of linolenic acid. Soy bean oil contains from 5 to about 9 per cent of linolenic acid, but this hardly seemed a likely source, because the amounts in the lard were greater than that in the soy bean oil and because Ellis reported only very small amounts of linolenic acid deposited as a result of feeding soy beans.

Of the various common oily domestic plants to which swine might have access in this country, only flaxseed is known to contain linolenic acid in

large quantities. The feeding to rats of 14.5 per cent linseed oil in the form of flaxseed resulted in the deposition of amounts of trienoic acids up to 27.59 per cent of the stored fats in the rat.

The melting points of the polybromides from the rat fat as well as the hog fat were unchanged by the addition of small proportions of known hexabromostearic acid from linseed oil. Likewise, the melting point of the known hexabromostearic acid was unchanged by the addition of small amounts of the polybromides. Furthermore, when the polybromides from the hog were mixed with the polybromides from the rat, there was no depression of the melting point. This would seem to indicate that the bromides were composed chiefly of 9,10,12,13,15,16-hexabromostearic acid from linolenic acid, and that the relatively low melting points observed were probably due to the presence of impurities which were not eliminated from the crystals. Due to the discrepancy in melting points, however, final proof of structure would need to be made by other means, as we did not have sufficient material for an elaborate series of fractional crystallizations. Knight, Jordan, and Swern (21), in studies on beef tallow, obtained ether-insoluble bromides melting at 170–171°, with no change in the melting point on the addition of hexabromostearic acid prepared from perilla oil, and considered this as evidence that the substances were identical. It may be that mutual solubility effects of the various bromides of polyunsaturated acids found in animal fats render the preparation of perfectly pure 9,10,12,13,15,16-hexabromostearic acid exceedingly difficult, if indeed this is the major constituent of the crystals obtained.

SUMMARY

1. Samples of "yellow" hog fat have been encountered occasionally which contain unusually large amounts of trienoic fatty acids. The amounts, calculated as linolenic acid, were found by spectrophotometric analysis to be as much as 11.4 per cent of the fatty acids present.

2. When linseed oil containing 45.78 per cent linolenic acid was fed to rats at a level of about 14.5 per cent of the ration by the incorporation of ground flaxseed, the amount of trienoic acids in the depot fat was found to be from 24.73 to 27.59 per cent of the fatty acids present. This was about 4 times the amount found in the ration.

3. It seems quite clear that animals such as the pig and the rat can deposit quite large amounts of trienoic acids in the depot fat, and that the amount deposited probably depends simply on the amount in the diet. This is somewhat in contrast to the view quite generally held, that these animals tend to remove the major portion of acids with more than two double bonds or to saturate them partially before depositing them in the stored fat.

4. The evidence indicates that "yellow" fat in swine may result from the feeding of flaxseed.

The authors wish to acknowledge the important technical assistance of Miss Helen Rezabek and Miss Marjorie Petheram in obtaining the analytical data.

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STUDIES ON INTERMEDIARY STEROID METABOLISM

II. COMPOUNDS ISOLATED FOLLOWING THE INCUBATION OF ANDROSTERONE AND ETIOCHOLAN-3(α)-OL-17-ONE WITH SURVIVING RABBIT LIVER SLICES

By JOHN J. SCHNEIDER AND HAROLD L. MASON

(From the Division of Biochemistry, Mayo Foundation, Rochester, Minnesota)

(Received for publication, April 15, 1948)

In the initial communication of this series (1) we described some experiments in which dehydroisoandrosterone was incubated with surviving rabbit liver slices in an aerobic environment. Under the conditions employed, this steroid was converted largely to Δ^5 -androstene-3(β), 17(α)-diol and in small yield to Δ^5 -androstene-3(β), 16(β), 17(α)-triol, which suggested that, while the urinary ketosteroids may be regarded as the excretion forms of other physiologically more active precursors, they are still susceptible to further metabolic change. In view of these results it appeared desirable to study *in vitro* the effect of various tissues on other representative urinary ketosteroids as a means of obtaining further information relative to their metabolic fate. It is the purpose of this paper to describe the isolation and characterization of the metabolic products formed when androsterone¹ and etiocholanolone were incubated with surviving rabbit liver slices under aerobic conditions. This study seemed particularly indicated in view of the demonstrated excretion of these two substances after the administration of dehydroisoandrosterone to human beings (2).

Procedure

The incubation procedure employed in these experiments was the same as that already described (1). In brief, slices were prepared from well chilled rabbit liver and were washed with, and later suspended in, aerated Krebs' phosphosaline buffer. To this system, which included no other factors, the steroid was added as the soluble sodium salt of its hemisuccinate in amounts sufficient to give a steroid to tissue ratio of from 1:300 to 1:400. The mixture was incubated with agitation for 6 hours at 38° under aerobic conditions.

The procedure used to isolate the products was substantially the same as that previously outlined, and different only in that the petroleum ether fractionation was not employed. In outline the method included, first, precipitation of the proteins with acid acetone, followed by removal of the

¹ We wish to thank Dr. E. Oppenheimer of Ciba Pharmaceutical Products, Inc., Summit, New Jersey, for a generous gift of androsterone.

acetone under reduced pressure. The aqueous residue was diluted, made alkaline, and extracted with ethylene dichloride in order to obtain the free steroid fraction. Further extraction of the aqueous phase following acidification gave a conjugated steroid fraction. The free steroid moiety was separated into ketonic and non-ketonic components. The free ketonic fraction was either chromatographed as such or first divided into digitonin-precipitable and digitonin-non-precipitable components. In each case the free non-ketonic fraction was saponified and the non-saponifiable fraction chromatographed. The crude conjugated fraction was saponified directly and the non-saponifiable fraction divided into ketonic and non-ketonic components.

TABLE I

Incubation of Androsterone and Etiocholan-3(α)-ol-17-one; Weights of Major Crude Fractions

Experiment No.	Androsterone	Etiocholan-3(α)-ol-17-one	Fractions obtained, mg.					
			Free steroid			Conjugated steroid		
			Total	Ketonic	Non-saponifiable, non-ketonic	Total	Non-saponifiable	
							Ketonic	Non-ketonic
A-1	1000		5071	105	1468	2767	140	282
A-2	1000		4042	108	1050	2560	298	518
E-1		1000	4842	121	1837	2463	6	132

Results

Table I indicates the total weight of the steroids incubated in each experiment, together with the weight of the major crude fractions.

Two experiments (Experiments A-1 and A-2) were carried out with the procedure described and with androsterone as a substrate. In each experiment the sodium androsterone hemisuccinate was incubated in divided amounts and the several extracts were combined before processing. Thus, in Experiment A-1, four 318 mg. portions of androsterone hemisuccinate (each equivalent to 250 mg. of free steroid) were incubated for 6 hours at a steroid to tissue ratio of approximately 1:400, while in Experiment A-2 three 424 mg. portions of androsterone hemisuccinate (each equivalent to 334 mg. of free steroid) were incubated for 6 hours at a steroid to tissue ratio of approximately 1:300. In each of the seven incubations the slices were suspended in 200 to 250 ml. of buffer.

The over-all recovery of crystalline compounds in the two experiments with androsterone averaged 37.3 per cent of the androsterone originally

incubated. Androsterone, which was recovered largely from the conjugated fraction, accounted for an average of 55.6 per cent of the weight of the steroids isolated. In both experiments, two metabolic products were identified. These were androstane-3(α),17(α)-diol, which was recovered largely from the conjugated non-saponifiable, non-ketonic fraction, and androstane-3,17-dione, which appeared exclusively in the free ketonic moiety. In one experiment, isoandrosterone was obtained in small amounts from the free ketonic fraction after treatment with digitonin. In addition, two other alcohols were obtained from the conjugated non-saponifiable, non-ketonic fractions but in amounts too small to permit

TABLE II

Distribution and Yield of Compounds Isolated after Incubation of Two 1000 Mg. Samples of Androsterone

Compound	Experiment No.	Fractions; weight in mg.				Yield, per cent of androsterone incubated
		Free steroid		Conjugated steroid, non-saponifiable		
		Ketonic	Non-saponifiable, non-ketonic	Ketonic	Non-ketonic	
Androstane-3(α), 17(α)-diol	A-1		11		100	11.1
	A-2		12		168	18.0
Androstane-3, 17-dione	A-1	6				0.6
	A-2	8			-	0.8
Isoandrosterone	A-1					
	A-2	5				0.5
Unknown Alcohol A	A-1				5	0.5
	A-2				3	0.3
" " B	A-1				8	0.8
	A-2				4	0.4
Androsterone	A-1	10		175		18.5
	A-2	14		218		23.2

identification. These have been designated as unknown Alcohols A and B. The formulas for the compounds identified are shown in Fig. 1. Table II indicates their distribution in the various fractions and the yields obtained.

In a single experiment (Experiment E-1) a total of 1000 mg. of etiocholan-3(α)-ol-17-one was incubated in three equal portions as the hemisuccinate. The incubation period was 6 hours and the steroid to tissue ratio was 1:300. The recovery of crystalline compounds was 50.8 per cent of the steroid added, and of this fraction 15.7 per cent was accounted for as etiocholanolone. Three compounds were isolated and identified. These were etiocholan-3(α),17(α)-diol and etiocholan-3(α),17(β)-diol, which were

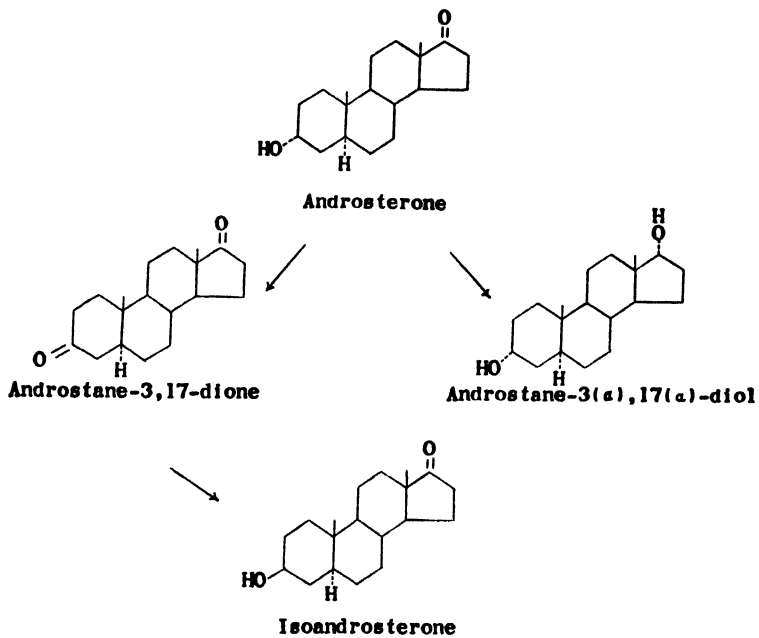


FIG. 1

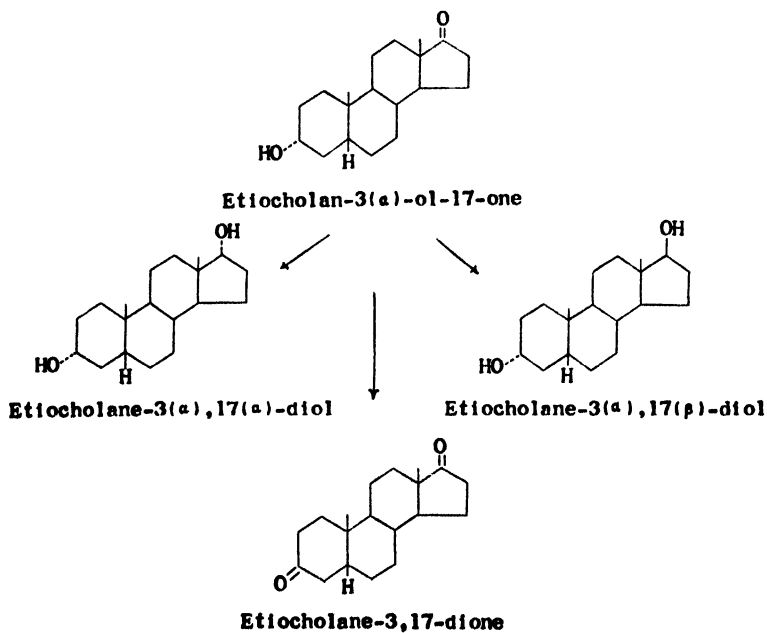


FIG. 2

recovered largely from the free, non-saponifiable, non-ketonic fraction, and etiocholane-3,17-dione, which was obtained from the free ketonic fraction. The formulas for these compounds appear in Fig. 2. The distribution of these compounds in the various fractions, together with the yields obtained, is indicated in Table III.

TABLE III
Distribution and Yield of Compounds Isolated after Incubation of 1000 Mg. of Etiocholan-3(α)-ol-17-one

Compound	Fractions; weight in mg.			Yield, per cent of etiocholanolone incubated
	Free steroid		Conjugated steroid, non-saponifiable, non-ketonic	
	Ketonic	Non-saponifiable, non-ketonic		
Etiocholan-3(α), 17(α)-diol		318	10	32.8
Etiocholan-3(α), 17(β)-diol		85		8.5
Etiocholan-3, 17-dione	15			1.5
Etiocholan-3(α)-ol-17-one	80			8.0

EXPERIMENTAL

All melting points were determined with the Fisher-Johns apparatus and are recorded as read.

Preparation of Androsterone and Etiocholanolone Succinates—These derivatives were prepared according to the procedure given for dehydroisoandrosterone in the previous publication (1). The yields varied from 83 to 93 per cent. Crystallization was best effected from ethyl acetate or methanol. Androsterone hemisuccinate melted at 186–187° (Ruzicka *et al.* (3) gave a value of 185–185.5°). Etiocholanolone hemisuccinate melted at 164–165°.

Isolation and Identification of Androstane-3(α), 17(α)-diol—In both Experiments A-1 and A-2, this compound was recovered largely from the conjugated non-ketonic, non-saponifiable fraction. It was usually eluted from the chromatographic column when the concentration of alcohol in benzene reached 0.3 to 0.6 per cent (by volume). It was readily crystallized from methanol to yield colorless needles which melted at 222–223°. A mixture with an authentic specimen of androstane-3(α), 17(α)-diol (m.p. 220–222°) melted at 220–222°. A sample treated with digitonin failed to form a precipitate. The diacetate, prepared with the aid of acetic anhydride and pyridine, melted at 162–163°.

Analysis of Diacetate— $C_{28}H_{48}O_4$. Calculated. C 73.36, H 9.64
Found. " 73.68, " 9.50

Isolation and Identification of Androstane-3,17-dione—In both Experiments A-1 and A-2, this compound appeared in the free ketonic fraction mixed with small amounts of androsterone and isoandrosterone. The recovery of the diketone was incomplete, due to the difficulty encountered in separating it from the associated hydroxy ketones, and in order to obtain a pure sample it was necessary to rechromatograph part of the free ketonic fractions. As would be expected, this compound was eluted from the column when the concentration of alcohol in carbon tetrachloride was of the order of 0.025 to 0.05 per cent. It was ultimately crystallized from dry ether and melted at 127–128°. A mixture with a known sample of androstane-3,17-dione (m.p. 127–128°) melted at 126–127°. It was further characterized by examination of its infra-red absorption spectrum² which agreed in all details with that obtained from an authentic sample.

Isolation and Identification of Isoandrosterone—This compound was identified as one of the products of Experiment A-2 and was isolated from that portion of the free ketonic fraction precipitated by digitonin. The best sample melted at 162–163°. A mixture with an authentic sample of isoandrosterone (m.p. 171–172°) melted at 164–165°. Infra-red absorption analysis² disclosed that the sample was an androsterone-isoandrosterone mixture.

Isolation and Identification of Androsterone—In Experiments A-1 and A-2, androsterone was obtained largely from the conjugated non-saponifiable ketonic fraction. It was usually eluted when the concentration of alcohol in carbon tetrachloride reached 0.05 to 0.1 per cent. It was crystallized from ethyl acetate and identified by its melting point and by the melting point of mixtures with authentic specimens.

Partial Characterization of Unknown Alcohols A and B—Both of these compounds were isolated from the conjugated non-saponifiable, non-ketonic fraction. Alcohol A, which was obtained in both Experiments A-1 and A-2, was crystallized from methanol and melted at 277–278°; $[\alpha]_D^{25} = +26.7^\circ \pm 1.2^\circ$ (0.320 per cent in ethanol). This compound was eluted from the chromatographic column when the alcohol concentration reached 0.7 to 1 per cent. It formed an acetate with acetic anhydride and pyridine which melted at 225–226°. Alcohol B was isolated from the same column, but not until the concentration of alcohol in benzene reached 10 to 15 per cent. After crystallization from methanol it melted at 233–234°; $[\alpha]_D^{25} = 0^\circ$ (0.249 per cent in ethanol). A mixture of the two alcohols melted at 205–215°.

Isolation and Identification of Etiocholan-3(α),17(α)-diol—This diol appeared largely in the free non-ketonic, non-saponifiable fraction and was

² We wish to thank Dr. Konrad Dobriner, Memorial Hospital, New York, for the infra-red analyses.

eluted when the concentration of alcohol in benzene reached 0.5 per cent. Its separation from the 17(β) isomer (which was present on the same column and which was eluted shortly after the 17(α) isomer) was due in part to a practice of eluting each component of a given column as completely as possible with the lowest practical concentration of alcohol. Crystallization from methanol yielded colorless plates which melted at 233–234°; $[\alpha]_D^{28} = +26.2^\circ \pm 1.5^\circ$ (1.29 per cent in ethanol). A mixture with an authentic sample of etiocholane-3(α),17(α)-diol (m.p. 232–233°) melted at 232–233°.

Analysis— $C_{19}H_{32}O_2$. Calculated, C 78.03, H 11.03; found, C 78.12, H 11.21

The diacetate, prepared with acetic anhydride and pyridine, melted at 125–126° (Butenandt *et al.* (4) gave a value of 232° as the melting point of the free diol and 124.5–125.5° as the melting point of its diacetate).

Isolation and Identification of Etiocholane-3(α),17(β)-diol—This as yet undescribed diol was eluted from the column when the concentration of alcohol in benzene was 0.7 per cent. It crystallized from aqueous methanol in the form of thin needles which melted sharply at 227–228°; $[\alpha]_D^{28} = 0^\circ$ (0.75 per cent in ethanol). A mixture with etiocholane-3(α),17(α)-diol (m.p. 233–234°) melted at 198–220°.

Analysis— $C_{19}H_{32}O_2$. Calculated, C 78.03, H 11.03; found, C 77.96, H 11.21

The 3(α) configuration was assigned because of its failure to form a precipitate when treated with digitonin, and because there was no reason to suspect that inversion had occurred at C-3. Oxidation with chromic acid in 90 per cent acetic acid yielded colorless needles which melted at 127–128°. A mixture with an authentic specimen of etiocholane-3,17-dione (m.p. 128–129°) melted at 127–128°. Thus, the only structural difference in the two diols was the configuration at C-17.

Isolation and Identification of Etiocholane-3,17-dione—This compound was obtained from the free ketonic fraction by washing the column with carbon tetrachloride containing 0.05 per cent alcohol. No difficulty was experienced in separating it from the associated etiocholanolone. It crystallized from dry ether in the form of colorless needles, and melted at 126–127°. A mixture with an authentic sample of etiocholane-3,17-dione (m.p. 127–128°) melted at 126–127°.

Analysis— $C_{19}H_{28}O_2$. Calculated, C 79.12, H 9.78; found, C 79.36, H 9.80

Isolation of Etiocholanolone—Etiocholanolone was obtained in Experiment E-1 from the free ketonic fraction only. Like androsterone, it was eluted from the column when the concentration of alcohol in carbon tetrachloride reached 0.05 to 0.1 per cent. It was identified in the manner described for androsterone.

Isolation and Identification of Cholesterol—Cholesterol was regularly isolated in all three experiments both from the free and the conjugated non-ketonic fractions. Its presence did not interfere with the isolation of other compounds of interest since it was eluted from the columns at an alcohol concentration well below that required to elute the dihydroxy compounds. It was crystallized from acetone and then melted at 148–149°.

Search for Acidic Fragments—Since the over-all recovery of neutral compounds was low in these experiments, a search was made on two occasions for possible acidic metabolic products. The alkaline aqueous solution remaining after extraction of the non-saponifiable material derived from the conjugated fraction was acidified and reextracted with ether. The dark brown extract so obtained was treated with an excess of diazomethane. After standing at room temperature for 12 hours, the solvent was evaporated, and the residue was taken up in benzene and chromatographed on acid-washed alumina. No crystalline substances were obtained by this procedure.

Comment

These experiments demonstrate that there exist in rabbit liver enzyme systems capable both of altering the functional groups of certain ketosteroids and of converting them into as yet unrecognized fragments. The reactions observed were of three general types. Quantitatively, the most important of these was the reduction of the C-17 carbonyl group to one or both epimeric alcohols. This was the predominating reaction with both androsterone and etiocholanolone in that it accounted for the largest share of the products isolated. The importance of this reaction was even more apparent in the case of dehydroisoandrosterone (1) in which from 43 to 69 per cent of the added steroid was converted to Δ^5 -androstene-3(β),17(α)-diol. It is of interest that both isomeric diols were obtained only in the case of etiocholanolone and that a careful search for the second isomer after androsterone incubation, and, in a recently repeated experiment, dehydroisoandrosterone, was unsuccessful.

These results suggest that reduction of the C-17 carbonyl group represents a general metabolic reaction of the 17-ketosteroids. The isolation from urine of a number of those steroids formed under the conditions employed in these *in vitro* experiments lends support to this view. Thus, although Δ^5 -androstene-3(β),17(α)-diol has not been isolated from normal urine, it has been recovered from the urine after administration of dehydroisoandrosterone to a human being (2), and in turn has led to an increased excretion of dehydroisoandrosterone after its subcutaneous injection into guinea pigs (5). Etiocholane-3(α),17(α)-diol has been recovered from the

urine of normal men (4) but its 17(β) isomer has not. Androstane-3(α),17(α)-diol, which was formed in significant amounts under *in vitro* conditions, has not been obtained from the urine of normal human beings to date, but has been isolated from the urine of a normal female after the administration of testosterone propionate (6).

A second reaction, which took place to a lesser extent, was that of oxidation of the C-3 hydroxyl group to the carbonyl group. This occurred during the incubation of both androsterone and etiocholanolone to give rise to the corresponding diketones. The normal occurrence of this reaction in human beings is attested by the previously reported isolation of androstane-3,17-dione and etiocholanone-3,17-dione from the urine of normal men and women (7). In addition, androstane-3,17-dione has been obtained from pig testis (8).

A third reaction, for which the evidence is less conclusive, appeared to consist of a reduction of the C-3 carbonyl group of the newly formed androstane-3,17-dione to yield one or both possible epimers. The evidence for this reaction is the isolation of isoandrosterone. However, since it is possible that the isoandrosterone isolated may have been present as a contaminant in the sample of androsterone incubated, it would be desirable to investigate this conversion further. The point would be most easily settled by separately incubating a sample of the intermediate, androstane-3,17-dione. Such experiments are now in progress.

Isoandrosterone has been isolated in small amounts from the urine of normal human beings (9), from the urine of a variety of patients with carcinoma (10, 11) and from the urine of patients with hyperfunctioning lesions of the adrenal cortex (12, 13).

These data, together with those already published, also show that the rates of hydrolysis of the three hemisuccinates in rabbit liver are distinctly different, as judged by the weight of crystalline compounds recovered from the conjugated fraction. The rate of hydrolysis of dehydroisoandrosterone hemisuccinate was the most rapid, and, by employing the same conditions, it was found that the rate of hydrolysis of androsterone hemisuccinate was the least rapid, while the rate of hydrolysis of etiocholanolone hemisuccinate occupied an intermediate position.

Of interest, too, is the demonstration that dehydroisoandrosterone or its metabolic products are comparatively resistant to those as yet undescribed enzyme systems that have as their point of action the steroid nucleus itself. In contrast to the 68 to 81 per cent recovery of crystalline compounds after the incubation of dehydroisoandrosterone, the recovery in the case of etiocholanolone was 50.8 per cent and in the case of androsterone 37.3 per cent.

SUMMARY

Sodium androsterone and etiocholan-3(α)-ol-17-one hemisuccinates were incubated with surviving rabbit liver slices under aerobic conditions for periods of 6 hours at 38°. An average of 37.3 per cent of the androsterone incubated was accounted for as the crystalline compounds androstane-3(α),17(α)-diol, androstane-3,17-dione, isoandrosterone, and two as yet unidentified alcohols. After the incubation of etiocholanolone the recovery of crystalline compounds was 50.8 per cent. These included etiocholan-3(α),17(α)-diol, etiocholan-3,17-dione, and a new steroid, etiocholan-3(α),17(β)-diol. The results have been discussed in relation to reported *in vivo* observations and with respect to ketosteroid metabolism in general.

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THE PREPARATION OF L-LEUCINE AND ITS BEHAVIOR IN SOME NON-AQUEOUS SOLVENTS

BY DUDLEY W. THOMAS AND CARL NIEMANN

(From the Gates and Crellin Laboratories of Chemistry,* California Institute of Technology, Pasadena)

(Received for publication, February 16, 1948)

Bergmann and Stein (1) have described a procedure for the isolation and purification of naturally occurring L-leucine based upon the precipitation and recrystallization of the slightly soluble salt of this amino acid with naphthalene- β -sulfonic acid. The product obtained was stated to be 97 per cent pure and to be free from methionine, a common contaminant of less pure preparations of L-leucine (2). We have found that L-leucine so prepared contains significant amounts of those amino acids which are oxidized by bromine in acid solution, and that a naphthalene sulfonate of superior purity can be obtained if the crude leucine is first treated with bromine water. The use of the preliminary bromine oxidation and of more dilute solutions than were used by Bergmann and Stein for the recrystallization of the naphthalene- β -sulfonate gave L-leucine preparations of exceptional purity when judged by qualitative tests and solubility determinations. Characteristic physical constants of L-leucine so prepared are given elsewhere in this paper.

In the determination of the optical rotation of α -amino acids in aqueous acid solutions, it has been assumed at times that the concentration of the aqueous acid is not critical provided sufficient acid has been added to assure formation of the amino acid cation. Dunn *et al.* (3) have pointed out that the specific rotation of solutions of L- and D-alanine in hydrochloric acid is dependent upon the hydrochloric acid concentration, and the data presented in Fig. 1 clearly show a similar dependency in the case of either hydrochloric or sulfuric acid solutions of L-leucine.

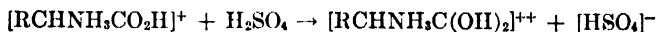
When solutions of L-leucine in 100 per cent sulfuric acid were allowed to stand at 25° for periods exceeding 1 month, no change in specific rotation was observed. Furthermore, the L-leucine recovered from these sulfuric acid solutions was indistinguishable from L-leucine not so treated. As either the lack of formation of the doubly charged cation, $[RCHNH_3C(OH)_2]^{++}$, or the stability of this ion, in respect to the loss of a proton from the α -carbon atom in sulfuric acid solutions, may have been responsible for the optical stability noted, the cryoscopic properties of sulfuric acid solutions of L-leucine were investigated. It was found that the freezing point

* Contribution No. 1175.

depression of these solutions was 2.2 times that of a non-electrolyte, which would indicate that although the reaction



is essentially complete there is relatively little ionization of the type



The effectiveness of the positively charged ammonium group in preventing appreciable protonation of the carboxyl group of the L-leucine cation in 100

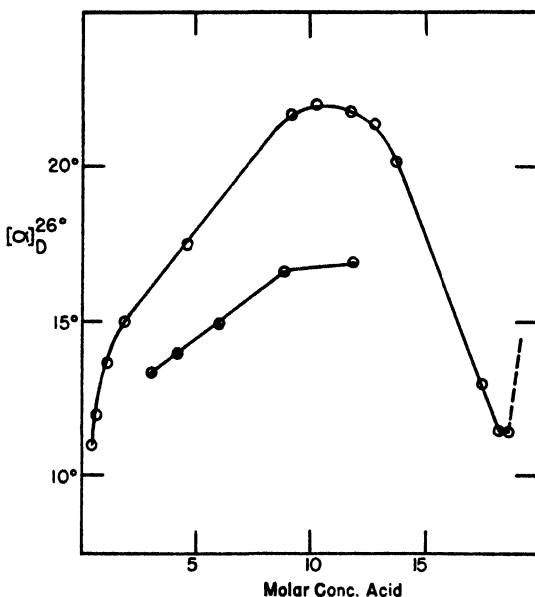


FIG. 1. Specific rotation of L-leucine in aqueous hydrochloric and sulfuric acid solutions. O, sulfuric acid; ●, hydrochloric acid.

per cent sulfuric acid can be appreciated when it is remembered that both acetic acid and monochloroacetic acid are completely ionized in this solvent (4).

It was observed that, when L-leucine was titrated with perchloric acid in glacial acetic acid solution (5), the specific rotation of the solution increased rapidly with added increments of perchloric acid until 1 equivalent of the acid had been added (Fig. 2). Kolthoff and Willman (6) have argued that a dipolar ion, such as glycine, would be expected to behave as a strong base in glacial acetic acid solutions because of the greater acid strength of acetic acid as compared to water and of an assumed near equivalence of the acid strengths of amino acid cation and acetic acid in solutions of the latter

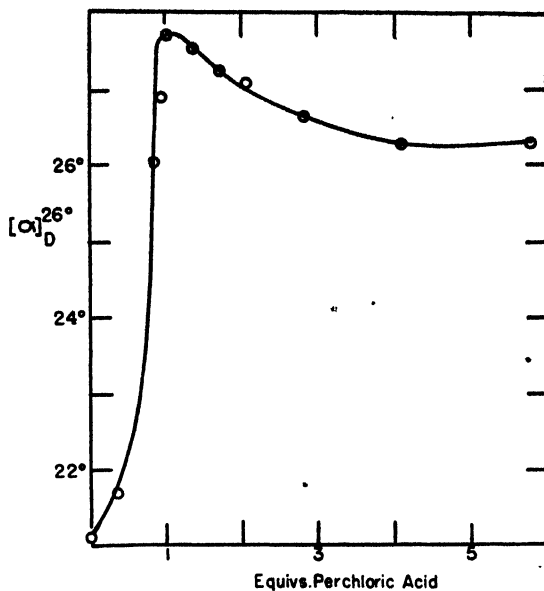


FIG. 2 Titration of L-leucine with perchloric acid in glacial acetic acid

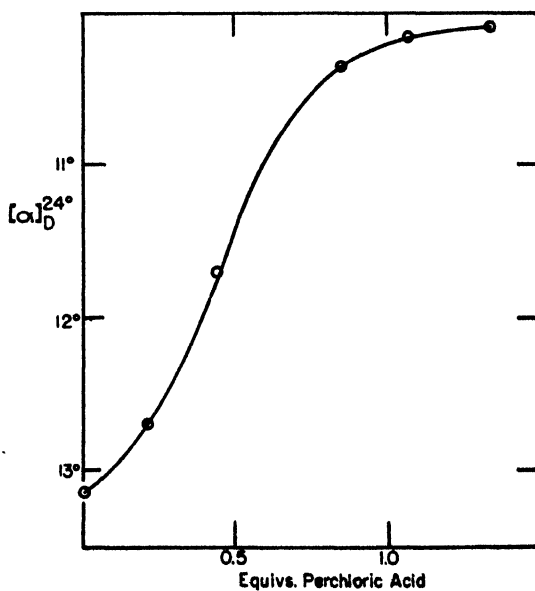


FIG. 3. Titration of d- α -phenylethylamine with perchloric acid in glacial acetic acid.

substance. These authors conclude, from conductivity measurements, that glycine dissolved in glacial acetic acid is present as $[\text{CH}_2\text{NH}_3\text{CO}_2\text{H}]^+[\text{Ac}]^-$ and that the degree of dissociation of this ion pair is of the same order as that of potassium or ammonium acetate in the same solvent. The observed change in the specific rotation of a glacial acetic acid solution of L-leucine upon the addition of perchloric acid in the same solvent and particularly the abrupt change in the trend of the specific rotation noted at the equivalence point can be interpreted to suggest negligible or limited dissociation of the ion pair $[\text{C}_4\text{H}_9\text{CHNH}_3\text{CO}_2\text{H}]^+[\text{Ac}]^-$ in glacial acetic acid.

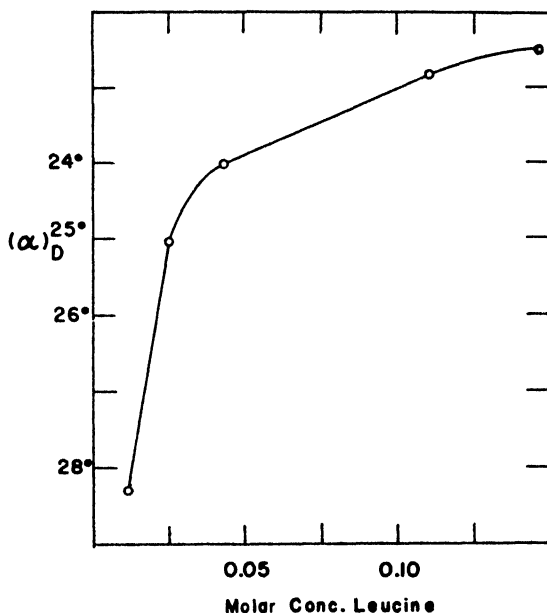


Fig. 4. Specific rotation of L-leucine in glacial acetic acid at 25°

If one assumes that the amino acid is completely ionized in glacial acetic acid solutions, the observed changes in the specific rotation of these solutions upon the addition of perchloric acid could be ascribed to the replacement of acetate ion by perchlorate ion in the undissociated ion pair. That such an effect is possible is demonstrated by the titration of a partially resolved sample of *d*- α -phenylethylamine in glacial acetic acid by perchloric acid in the same solvent (Fig. 3). In the latter case there can be no doubt that the α -phenylethylamine is completely ionized in glacial acetic acid solutions. Although rigorous evidence as to the nature of the ion species present in a glacial acetic acid solution of L-leucine is lacking, present knowledge (4, 7-9) would appear to offer little support to the

view that the amino acid can exist in glacial acetic acid solutions as the dipolar ion.

In the course of these studies, it was observed that the specific rotation of L-leucine in glacial acetic acid is strikingly dependent not only upon temperature but also upon the amino acid concentration (Fig. 4). Furthermore, it was observed that lowering the molal freezing point of solutions of L-leucine in glacial acetic acid varies with the amino acid concentration, as is shown in Fig. 5. These observations suggest that even at relatively low concentration there is extensive association of the ion pairs present in a glacial acetic acid solution of L-leucine.

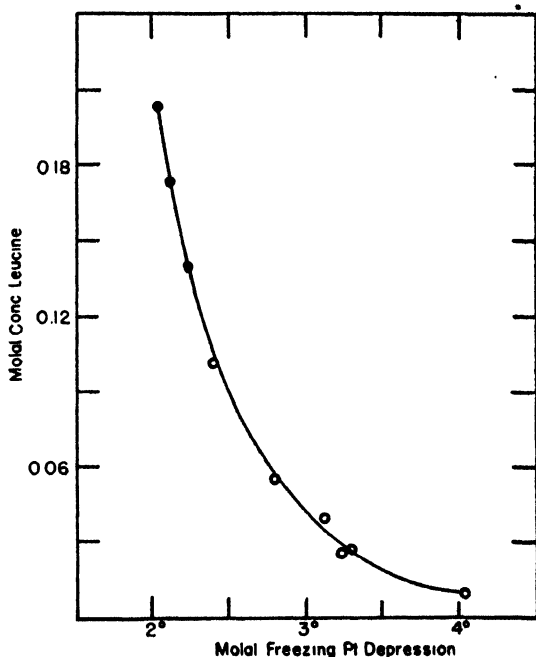


FIG. 5. Freezing point depression of solutions of L-leucine in glacial acetic acid

EXPERIMENTAL

Amperometric Titration of Leucine Preparations (10)—The titration of 100 mg. samples gave the following values, expressed as moles of Br_2 consumed per gm. of amino acid: a technical leucine (Lemke), 1.31×10^{-3} ; a purified leucine (Hoffmann-La Roche), 3.66×10^{-4} ; and leucine prepared by the method of Bergmann and Stein (1), 1.11×10^{-5} . Recrystallization of the naphthalene- β -sulfonate, beyond that advocated by Bergmann and Stein, did not prove effective in removing bromine-oxidizable impurities.

Preparation of L-Leucine—Saturated bromine water was added, slowly

and with stirring, to a 10 per cent (weight per volume) solution of technical leucine (Lemke) in 1 M¹ hydrochloric acid until the color persisted for 20 minutes after the last addition. The excess bromine was destroyed with sodium bisulfite prior to the addition of naphthalene- β -sulfonic acid as directed by Bergmann and Stein (1). The naphthalene- β -sulfonate, m.p. 189–191.5°, $[\alpha]_D^{25} = +13.3^\circ \pm 0.1^\circ$, 4.3 per cent (weight per volume) in methyl cellosolve, was recrystallized four times from a 7.5 per cent (weight per volume) aqueous solution and the L-leucine, obtained by the decomposition (1) of the four times recrystallized naphthalene- β -sulfonate, m.p. 191–192°, $[\alpha]_D^{25} = +13.2^\circ \pm 0.1^\circ$, 3.8 per cent (weight per volume) in methyl cellosolve, was recrystallized twice from 33 per cent (by volume) aqueous ethanol. The yield of the twice recrystallized L-leucine, based upon the weight of the starting material, varied between 10 and 14 per cent. Qualitative tests for the presence of sulfur (11) and of tyrosine (12) in the twice recrystallized L-leucine were negative. Examination of the absorption spectra of a 0.4 per cent (weight per volume) aqueous solution of the amino acid revealed no specific absorption in the 260 to 270 μ region. Analysis for total nitrogen gave 10.60 ± 0.09 per cent or 99.3 ± 0.8 per cent of theory.

Solubility of L-Leucine in Water—An excess of twice recrystallized L-leucine was equilibrated with redistilled water at $25.05^\circ \pm 0.05^\circ$. Aliquots were withdrawn over a period extending from 5 to 30 days and the solvent was evaporated at 105° . Seventeen determinations gave a mean value of 2.152 ± 0.006 gm. per 100 gm. of water for the solubility at 25° . The undissolved leucine remaining from the above series of solubility measurements was utilized for a second series of measurements, and in this instance twelve determinations, conducted as described above, gave a mean value of 2.15 ± 0.01 gm. per 100 gm. of water at 25° . The value of 2.15 ± 0.01 gm. per 100 gm. of water for the solubility of L-leucine in water at 25° obtained above is to be compared with the value of 2.19 gm. per 100 gm. of water at 25° given by Stoddard and Dunn (9) and the value of 2.20 gm. per 100 gm. of water at 25° reported by Hlynka (13).

Optical Rotation of L-Leucine in Aqueous Systems—The optical rotation of the twice recrystallized L-leucine was determined in 6.02 M hydrochloric acid, with the leucine concentration varying from 2.0 to 5.0 per cent (weight per volume) and the temperature from 18– 35° . The specific rotation of L-leucine in 6.02 M hydrochloric acid was found to be independent of the leucine concentration between the limits studied. The variation of the specific rotation with temperature was found to be linear within the above limits, the temperature coefficient having a value of 0.07° per 1° , in reason-

¹ Molar and molal are used in a conventional sense only and are not to be construed as indicative of the actual molecular species that may be present in solution.

able agreement with the value of 0.063° per 1° reported by Stoddard and Dunn (9) for L-leucine in 6.08 M hydrochloric acid.

The dependence of the specific rotation of L-leucine upon the concentration of the aqueous hydrochloric or sulfuric acid used as a solvent was studied, and the data obtained are presented in Fig. 1. In order to compare values for the specific rotation of L-leucine in aqueous hydrochloric acid solutions reported by others with those obtained in this study, the former values were interpolated to a temperature of 25° and a hydrochloric acid concentration of 6.0 M. The values so obtained are given in Table I.

Behavior of L-Leucine in Sulfuric Acid—The optical stability of L-leucine in 100 per cent sulfuric acid was determined by preparing a 0.1524 M so-

TABLE I
Specific Rotation of L-Leucine in 6.0 M Hydrochloric Acid at 25°

Author	Value in literature	Interpolated value*
	<i>degrees</i>	<i>degrees</i>
Bergmann and Stein (1)	15.33 (21% HCl, 24°)	15.24
Stoddard and Dunn (9)	15.21 (6.08 M HCl, 25°)	15.16
Dunn and Courtney (14)	15.1 (6.0 " " 25.9°)	15.03
Thomas and Niemann (this paper)	14.85 (6.02 " " 25°)	14.84

* Interpolated to 6.00 M HCl and 25° .

lution of this amino acid in 100 per cent sulfuric acid and observing the specific rotation of the solution immediately after preparation and after it had stood at room temperature in an air-tight container for 8 weeks. No significant change was observed. The solution was then diluted with water, and the amino acid was recovered by precipitation with naphthalene- β -sulfonic acid and compared with L-leucine naphthalene- β -sulfonate of known purity. The determination of the mixed melting point, nitrogen content, and specific rotation failed to disclose any differences.

The cryoscopic properties of L-leucine in 100 per cent sulfuric acid were studied by the method described by Hammett and Deyrup (15). The freezing point depression of 0.03 to 0.12 molal solutions of L-leucine in 100 per cent sulfuric acid was found to be $13.5^\circ \pm 0.2^\circ$ per mole of amino acid or approximately 2.2 ± 0.05 times the depression caused by a non-electrolyte.

Behavior of L-Leucine in Glacial Acetic Acid—The specific rotation of glacial acetic acid solutions containing varying amounts of perchloric acid and 1.0 gm. of twice recrystallized L-leucine per 50 gm. of solution was determined at 26° . These data are given in Fig. 2. For comparative

purposes the specific rotation of solutions of *d*- α -phenylethylamine, $[\alpha]_D^{24} = +37.6^\circ$, containing 0.50 gm. of the amine in 25 ml. of acetic acid-perchloric acid of varying perchloric acid concentration, was determined at 24° (Fig. 3). The freezing point depression of solutions of twice recrystallized L-leucine in glacial acetic acid was determined with the aid of a Beckmann thermometer. The results of these cryoscopic measurements are presented in Fig. 5.

SUMMARY

A procedure for the preparation of L-leucine is described and certain physical properties, useful for establishing the identity or purity, have been redetermined. The behavior of L-leucine in sulfuric acid and glacial acetic acid solutions has been investigated.

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STEROIDS DERIVED FROM BILE ACIDS

VIII. CATALYTIC HYDROGENATION OF METHYL 3(α)-HYDROXY-12-KETO- $\Delta^{9,11}$ -CHOLENATE AND RELATED COMPOUNDS*

By BERNARD F. MCKENZIE, VERNON R. MATTOX, AND
EDWARD C. KENDALL

(From the Division of Biochemistry, Mayo Foundation, Rochester, Minnesota)

(Received for publication, April 19, 1948)

The reduction of the 12-carbonyl group of methyl 3(α)-hydroxy-12-keto- $\Delta^{9,11}$ -cholelate to a hydroxyl group has been reported as a step in the preparation of methyl 3,9-epoxy- Δ^{11} -cholelate (1). In the course of an extended investigation of this reduction with hydrogen and Adams' platinum oxide catalyst (2), several factors were found which influence the rate and course of the hydrogenation. The results of this inquiry and the hydrogenation and hydrogenolysis of some closely related compounds are discussed in this paper.

Solvent

In neutral methanol or ethanol neither the carbonyl group nor the double bond of methyl 3(α)-hydroxy-12-keto- $\Delta^{9,11}$ -cholelate was reduced with hydrogen and Adams' platinum oxide catalyst. In acetic acid and with not more than 3 gm. of platinum oxide per mole of steroid, hydrogen was absorbed until 1 mole had been taken up. The same result was obtained in mixtures of acetic acid with methanol or ethanol, but the rate of reduction was decreased in the presence of acetic acid-ethanol, 1:3.

The nature of the activation of the carbonyl group which permits absorption of hydrogen is not known, but the necessity for a certain concentration of hydrogen ion is clearly evident. Dr. Jacob van de Kamp, Merck and Company, Research Laboratories, has shown that methyl 3(α)-hydroxy-12-keto- $\Delta^{9,11}$ -cholelate in methanol absorbed 1 mole of hydrogen rapidly if a small amount of hydrogen chloride was present.¹ Utilization of hydrogen then stopped. Determination of the amount of unsaturated ketone by the extinction coefficient of the hydrogenated products indicated that less than 2 per cent of the starting material was present.

The addition of small amounts of water (6 per cent) to methanol which contained 0.05 N hydrogen chloride reduced the rate of hydrogenation slightly, but with the same concentration of hydrogen chloride 16 per cent

* This work was supported in part by a grant from the Research Corporation, New York.

¹ van de Kamp, J., personal communication.

of water appreciably decreased the rate of utilization of hydrogen. With 1.0 N hydrogen chloride and 16 per cent of water the rate of hydrogenation was markedly reduced.

With acetic acid as solvent 0.004 N hydrogen chloride increased the rate of reduction, but 0.10 N hydrogen chloride suppressed the absorption of hydrogen.

Platinum Catalyst

The reduction of the 12-carbonyl group in methyl 3(α)-hydroxy-12-keto- $\Delta^9,11$ -cholenate was dependent on the order of addition of the ketosteroid to the solvent. If the ketosteroid and platinum oxide were added to the solvent at the same time, hydrogenation proceeded satisfactorily. If the platinum oxide was first reduced, hydrogen was not absorbed after addition of the steroid.

Other evidence for the loss of activity of the catalyst was afforded by the inability of the platinum to reduce more than the amount of steroid initially present. After reduction of the 12-keto group of the starting material the platinum catalyst was not effective for reduction of further amounts of the same product.

Not only the rate but also the extent of reduction of methyl 3(α)-hydroxy-12-keto- $\Delta^9,11$ -cholenate was dependent on the amount of platinum catalyst. With not more than 2 gm. of platinum oxide per mole of steroid, the catalyst appeared to be exhausted after reduction of the 12-ketone, but with larger amounts of platinum (from 5 to 150 gm.) reduction of the carbonyl group was invariably followed by hydrogenolysis of the 12-hydroxyl group and formation of methyl 3(α)-hydroxy- $\Delta^9,11$ -cholenate.

Selenium

In almost all of the reductions of methyl 3(α)-hydroxy-12-keto- $\Delta^9,11$ -cholenate it is probable that traces of selenium were present. In some experiments the amount of selenium was so great that utilization of hydrogen proceeded only for a fraction of 1 molar equivalent. It was then found that addition of more platinum oxide without removal of the first portion was not always satisfactory. However, if the poisoned platinum was removed, addition of 2 or 3 gm. of platinum oxide per mole of steroid usually brought about absorption of 1 molar equivalent of hydrogen. The poisoning of the catalyst with selenium occurred in the earlier experiments before a satisfactory method for its removal had been devised, but it is probable that also in many of the later large scale preparations traces of selenium were present. This fact is pointed out, since it may seem likely that selenium may be an important factor in the reduction and that not only the rate but the course of reduction was modified by selenium.

However, the results of an extended investigation indicate that, except with regard to the rate of hydrogenation, selenium does not modify the utilization of hydrogen. In no instance was the sequence of reduction changed by the presence of selenium. Invariably there was reduction of the 12-carbonyl group to an (α)-hydroxyl or (β)-hydroxyl group, hydrogenolysis to form methyl 3(α)-hydroxy- $\Delta^{9,11}$ -cholenate, and finally reduction of the double bond to give methyl lithocholate. For the reduction of the $\Delta^{9,11}$ bond fresh platinum oxide was required.

A comparison of the results obtained with varying amounts of platinum with many samples of compounds which contained the 12-keto- $\Delta^{9,11}$ grouping clearly indicates that traces of selenium exert a favorable influence. This is because the activity of the platinum is modified to such an extent that hydrogenolysis of the 12-hydroxyl group does not occur. With acetic acid-ethanol as solvent, as little as 3 gm. of platinum oxide per mole of steroid could cause hydrogenolysis of 5 to 10 per cent of the 12-hydroxyl group. This was not observed even over many hours if the amount of platinum was reduced to 2 gm. per mole of steroid. However, if traces of selenium were present, from 5 to 10 gm. of platinum oxide per mole of steroid could be used; the reduction proceeded rapidly and stopped abruptly after utilization of 1 mole of hydrogen.

Hydrogenation of Methyl 3(α)-Hydroxy-12-keto- $\Delta^{9,11}$ -cholenate

Hydrogenation of methyl 3(α)-hydroxy-12-keto- $\Delta^{9,11}$ -cholenate in ethanol-acetic acid, 1:1, yielded an almost quantitative reduction of the 12-carbonyl group and gave a mixture of 12(α)-hydroxy and 12(β)-hydroxy compounds. The extinction coefficient indicated the presence of not more than 1.6 per cent of unsaturated ketone. Chromatographic separation was not satisfactory, but the determination of the specific rotation indicated that the proportion between the α and β forms was about 1:4. The larger amount of the 12(β) epimer with the hydroxyl group cis to the methyl group at C₁₃ is in keeping with the mechanism suggested by Linstead and associates (3).

Direct isolation of either form of the dihydroxy compounds epimeric at C₁₂ or of the acids after hydrolysis of the ester eluded diligent and extended effort. A derivative, methyl 3(α),12-diacetoxy- $\Delta^{9,11}$ -cholenate, was separated and crystallized until the melting point and specific rotation were constant. This compound was different from the known methyl 3(α),12-(α)-diacetoxy- $\Delta^{9,11}$ -cholenate (4), and hydrolysis with alkali yielded a crystalline acid which was different from 3(α),12(α)-dihydroxy- $\Delta^{9,11}$ -cholenic acid (4). However, both the new dihydroxy compound and its diacetate in methanol with a trace of hydrogen chloride gave an almost quantitative yield of methyl 3(α)-hydroxy-12(α)-methoxy-

$\Delta^{9,11}$ -cholenate (4, 5), and in acetic acid with a trace of sulfuric acid the dihydroxy acid was converted into 3(α)-hydroxy-12(α)-acetoxy- $\Delta^{9,11}$ -cholenic acid (4, 5). We may conclude, therefore, that the compounds under discussion are 3(α), 12(β)-dihydroxy- $\Delta^{9,11}$ -cholenic acid and methyl 3(α), 12(β)-diacetoxy- $\Delta^{9,11}$ -cholenate.

Hydrogenation of Methyl 3(α)-Acetoxy-12-keto- $\Delta^{9,11}$ -cholenate

Reduction of the 12-carbonyl group of methyl 3(α)-acetoxy-12-keto- $\Delta^{9,11}$ -cholenate (6) occurred under the same conditions described for the 3(α)-hydroxy compound with the absorption of 1 mole of hydrogen. Absorption of hydrogen then stopped and when the reduction products were separated by adsorption on aluminum oxide and elution, they were shown to be methyl 3(α)-acetoxy-12(α)-hydroxy- $\Delta^{9,11}$ -cholenate (5, 6) and the epimeric 12(β)-hydroxy compound. The presence of the 3(α)-acetyl group compared with the 3(α)-hydroxyl group appeared to increase the ratio of the 12(α) epimer to the 12(β) compound from 1:4 to 2:3.

Hydrogenolysis

Hydrogenolysis of a group adjacent to a double bond has been observed in numerous compounds (7), and it was found that the substituent at C₁₂, activated by the double bond C₉-C₁₁, could be quantitatively replaced with hydrogen in the presence of Adams' platinum oxide catalyst. With 2 gm. or less of platinum oxide per mole of 12-ketosteroid only 1 mole of hydrogen was utilized. With 3 gm. or more of platinum oxide reduction of the carbonyl group was rapid and in addition hydrogenolysis of the hydroxyl group at C₁₂ occurred. Methyl 3(α)-hydroxy-12-keto- $\Delta^{9,11}$ -cholenate and both methyl 3(α), 12(α)-dihydroxy- $\Delta^{9,11}$ -cholenate and the epimeric 12(β)-hydroxy compound were reduced to methyl 3(α)-hydroxy- $\Delta^{9,11}$ -cholenate (6) in acetic acid-ethanol, 1:1, in the presence of 100 gm. of platinum catalyst per mole of steroid. Under similar conditions methyl 3(α)-hydroxy-12(α)-acetoxy- $\Delta^{9,11}$ -cholenate (4, 5), methyl 3(α), 12(α)-diacetoxy- $\Delta^{9,11}$ -cholenate (4, 5), methyl 3(α)-acetoxy-12-keto- $\Delta^{9,11}$ -cholenate (5, 6), and methyl 3(α)-hydroxy-12(α)-methoxy- $\Delta^{9,11}$ -cholenate (4, 5) were reduced to methyl 3(α)-hydroxy- $\Delta^{9,11}$ -cholenate (6) (XIII)² or the acetate of XIII.

It has already been reported that in acetic acid methyl 3,9-epoxy- Δ^{11} -cholenate is very rapidly reduced to methyl 3(α)-hydroxy- $\Delta^{9,11}$ -cholenate (6). The compound mentioned last or its acetate appears to be the first product formed by hydrogenation with each of the eight compounds

² See Table I.

discussed in this section. In no instance did reduction of the double bond C_9-C_{11} occur before hydrogenolysis of the group at C_{12} .

EXPERIMENTAL

In all experiments the pressure of hydrogen was about 1 atmosphere and was determined with a mercury manometer. All samples of Adams' platinum oxide were prepared at 450° and therefore were probably hydrated. In this paper they will be designated PtO_2 . All melting points

TABLE I
Compounds Hydrogenated

Compound	Compound No.	Bibliographic reference No.
Methyl 3(α)-hydroxy-12-keto- $\Delta^{9,11}$ -cholenate	I	(1)
3(α)-Hydroxy-12-keto- $\Delta^{9,11}$ -cholenic acid	II	(1)
Methyl 3(α), 12(α)-diacetoxy- $\Delta^{9,11}$ -cholenate	III	(4, 5)
" 3(α), 12(β)-diacetoxy- $\Delta^{9,11}$ -cholenate	IV	*
" 3(α), 12(α)-dihydroxy- $\Delta^{9,11}$ -cholenate	V	(4, 5)
3(α), 12(β)-Dihydroxy- $\Delta^{9,11}$ -cholenic acid	VI	*
Methyl 3(α)-acetoxy-12-keto- $\Delta^{9,11}$ -cholenate	VII	*, (6)
" 3(α)-acetoxy-12(α)-hydroxy- $\Delta^{9,11}$ -cholenate	VIII	(5, 6)
Methyl 3(α)-acetoxy-12(β)-hydroxy- $\Delta^{9,11}$ -cholenate	IX	*
Methyl 3(α)-hydroxy-12(α)-methoxy- $\Delta^{9,11}$ -cholenate	X	(4, 5)
Methyl 3(α)-hydroxy-12(α)-acetoxy- $\Delta^{9,11}$ -cholenate	XI	(4, 5)
3(α)-Hydroxy-12(α)-acetoxy- $\Delta^{9,11}$ -cholenic acid	XII	(4, 5)
Methyl 3(α)-acetoxy- $\Delta^{9,11}$ -cholenate	XIII	(6)

* This paper.

were determined on the Fisher-Johns apparatus. The compounds hydrogenated are listed in Table I.

Hydrogenation of Methyl 3(α)-Hydroxy-12-keto- $\Delta^{9,11}$ -cholenate (1) (I)

In Methanol with HCl—A solution of 4.02 gm. of I in 50 cc. of methanol was shaken in an atmosphere of hydrogen in the presence of 50 mg. of PtO_2 . In 12 minutes 4 cc. of hydrogen in excess of that required by the catalyst were absorbed, but no further uptake was observed in 3 hours. After the addition of 0.2 cc. of concentrated aqueous HCl, reduction proceeded satisfactorily. In 20 hours 0.98 mole of hydrogen was absorbed.

In Methanol with 0.05 N HCl—1 mole of I was dissolved in 1200 cc. of

methanol and filtered through 10 gm. of activated carbon. 5 cc. of concentrated aqueous HCl to give 0.05 N HCl were added. 2 gm. of PtO_2 were used. The absorption of hydrogen, expressed as per cent of 1 mole, and the time required in seven experiments were as follows:

Time, hrs., min.	17.30	6.30	7.50	24.00	26.00	24.00	33.00
%	99	99	99	100	100	99	100

In Methanol with Varying Amounts of Water and Acid—The influence of increasing percentages of water and of hydrogen chloride is shown in Table II.

TABLE II
Influence of Water and Acid on Rate of Hydrogenation in Methanol

Time	Solution*	H ₂ absorbed
hrs.		mole
1	A	0.43
	B	0.32
	C	0.19
	D	0.10
2	A	0.59
	B	0.50
	C	0.34
	D	0.19
4	A	0.72
	B	0.66
	C	0.52
	D	0.34
8	A	0.84
	B	0.80
	C	0.72
	D	0.58
22	A	1.00
21	B	0.98
23	C	0.99
23	D	0.98

* Each of the four solutions was prepared with 4.02 gm. of the same sample of I, 30 mg. of PtO_2 , and 12 cc. of methanol. The necessary amounts of water and aqueous HCl (12 N or 1 N) were added to give the following percentages of water and normalities of acid.

Solution	H ₂ O per cent	HCl N
A	2	0.05
B	6	0.05
C	16	0.05
D	16	1.00

In Ethanol and with 0.05 N HCl—A solution of 4.02 gm. of I in 50 cc. of ethanol (95 per cent) was shaken in an atmosphere of hydrogen in the presence of 50 mg. of PtO_2 . In 20 minutes 4 cc. of hydrogen in excess of that required by the catalyst were absorbed, but no further uptake was recorded in the next hour. Reduction proceeded satisfactorily after addition of 0.2 cc. of concentrated aqueous HCl and 1 mole of hydrogen was absorbed in 12 hours. The uptake of hydrogen ceased at this point.

In Acetic Acid—48.6 gm. of I with 250 mg. of PtO_2 (2.3 gm. per mole of steroid) in 250 cc. of acetic acid absorbed 1.01 moles of hydrogen in 23 hours.

121 gm. of I in 500 cc. of acetic acid with 500 mg. of PtO_2 (1.66 gm. per mole) absorbed 1.01 moles of hydrogen in 23 hours.

In Acetic Acid with Small Amounts of HCl—4.02 gm. of I in 50 cc. of acetic acid with 30 mg. of PtO_2 and 1 cc. of 0.10 N aqueous HCl (0.002 N) absorbed 0.99 mole of hydrogen in 16 hours. In a similar experiment with 50 mg. of PtO_2 and 2 cc. of 0.10 N HCl (0.004 N), 0.92 mole of hydrogen was absorbed in 5 hours and 1.04 moles after 21 hours.

In Acetic Acid with 0.10 N HCl—In three experiments 4.02 gm. of I in 50 cc. of acetic acid and 0.41 cc. of concentrated aqueous HCl in the presence of 50 mg. of PtO_2 absorbed 5, 11, and 11 per cent of 1 molar equivalent of hydrogen respectively in 1 hour. The presence of 0.10 N HCl suppressed the absorption of hydrogen.

In Acetic Acid-Ethanol—1 mole of I absorbed 1 mole of hydrogen in each of three experiments. 600 cc. of acetic acid, 600 cc. of ethanol (95 per cent), and 2.0 gm. of PtO_2 were used. 33, 45, and 49 hours respectively were required.

Acetic Acid-Ethanol As Solvent for Reduction of VII—The influence of varying proportions of acetic acid-ethanol on the reduction of the carbonyl group of methyl 3(α)-acetoxy-12-keto- $\Delta^{9,11}$ -cholenate is shown in Table III.

Influence of Selenium on Hydrogenation—98 gm. (0.252 mole) of 3(α)-hydroxy-12-keto- $\Delta^{9,11}$ -cholenic acid (II) (4) with 500 mg. of PtO_2 (2 gm. per mole) in 400 cc. of acetic acid and 400 cc. of ethanol (95 per cent) absorbed 0.05 mole of hydrogen in 4 hours. The solution was filtered and 500 mg. of PtO_2 were added. 0.07 mole of hydrogen was absorbed in 8 hours. The solution was filtered, and 500 mg. of PtO_2 were added. 0.22 mole of hydrogen was absorbed in 2.2 hours and 0.99 mole after 31 hours. The first portions of platinum contained selenium.

Removal of Selenium from Platinum—Selenium is not removed from platinum by solution of the metal in aqua regia followed by precipitation of the chloroplatinic acid with ammonium salts. Furthermore, selenium is not removed when PtO_2 is formed by fusion of salts of chloroplatinic acid

with sodium nitrate. For the removal of selenium from platinum we have found that the method devised for the determination of selenium in soil through volatilization of the tetrabromide is satisfactory and can be applied conveniently (8). The accumulated platinum residues separated after catalytic reductions were dissolved in aqua regia, and the water and acids were boiled off. Concentrated hydrobromic acid and 1 to 2 cc. of bromine were added and the solution was boiled. The distillate was condensed in an all-glass apparatus and was tested for the presence of selenium by addition of SO_2 . It was sometimes necessary to repeat the distillation with one or more portions of hydrobromic acid and bromine.

After all selenium had been removed as shown by the absence of selenium in the distillate, the remaining hydrobromic acid was boiled off and the

TABLE III

*Hydrogenation of Methyl 3(α)-Acetoxy-12-keto- $\Delta^{9,11}$ -cholenate (VII) in Varying Amounts of Acetic Acid-Ethanol**

Solution		H_2 absorbed, per cent of 1 mole of H_2			
Acetic acid	Ethanol	30 min.	60 min.	90 min.	120 min.
cc.	cc.				
6	2	43	69	88	101
4	4	43	73	93	103
2	6	24	46	64	77

* 0.001 mole of the same sample of steroid and 10 mg. of PtO_2 were used in each experiment.

bromoplatinic acid was precipitated with ammonium chloride. Fusion of this salt with sodium nitrate as suggested by Voorhees and Adams (2) invariably yielded PtO_2 with a high catalytic activity.

Influence of Amount of Platinum—Several examples of the absorption of more than 1 mole of hydrogen per mole of steroid are included in the experimental results. In no instance has utilization of hydrogen exceeded 1 mole in the presence of 2 gm. of PtO_2 (Fig. 1).

Methyl 3(α),12(β)-Diacetoxy- $\Delta^{9,11}$ -cholenate (IV) (4, 5) from Methyl 3(α)-Hydroxy-12-keto- $\Delta^{9,11}$ -cholenate (I)(1)—40.2 gm. (0.10 mole) of I were dissolved in 250 cc. of acetic acid and 250 cc. of ethanol (95 per cent) and shaken in an atmosphere of hydrogen in the presence of 1 gm. of PtO_2 . 1.06 moles of hydrogen were absorbed in 1 hour and 36 minutes.

The platinum was removed by filtration and the solution was concentrated to a small volume. 500 cc. of benzene were added and the mixture was washed with water. The benzene was removed under reduced pressure, and the ester was acetylated in 100 cc. of pyridine and 100 cc. of

acetic anhydride at room temperature for 15 hours. Benzene was added and the mixture was shaken with water, with dilute hydrochloric acid, and with water. The benzene was removed under reduced pressure and the methyl 3(α),12(β)-diacetoxy- $\Delta^{9,11}$ -cholenate was crystallized three times

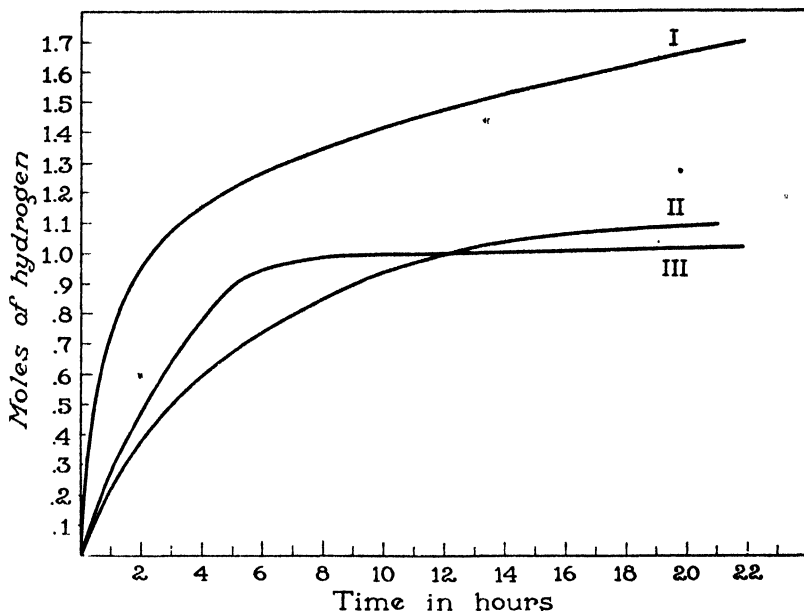


FIG. 1. Curve I, 0.2010 gm. (0.0005 mole) of methyl 3(α)-hydroxy-12-keto- $\Delta^{9,11}$ -cholenate (I) was reduced in 10 cc. of acetic acid with 50 mg. of PtO_2 (100 gm. per mole). Absorption of hydrogen proceeded until 1.99 moles had been utilized in 70 hours. Curve II, 4.02 gm. (0.01 mole) of I in 25 cc. of acetic acid and 25 cc. of ethanol with 30 mg. of PtO_2 (3 gm. per mole). Curve III, 0.404 gm. (0.001 mole) of I in 2.5 cc. of acetic acid, 2.5 cc. of ethanol with 2 mg. of PtO_2 (2 gm. per mole).

from 120 cc. portions of methanol. The product weighed 27.8 gm. and melted at $106.8\text{--}107^\circ$. $[\alpha]_D^{25} = +3^\circ \pm 2^\circ$ ($c = 1$ in methanol).

*Analysis*²— $\text{C}_{29}\text{H}_{44}\text{O}_6$. Calculated, C 71.28, H 9.07; found, C 71.16, H 9.26

3(α),12(β)-Dihydroxy- $\Delta^{9,11}$ -cholenic Acid (VI) from Methyl 3(α),12(β)-diacetoxy- $\Delta^{9,11}$ -cholenate (IV)—13.44 gm. of IV were dissolved at room temperature in 200 cc. of methanol and 50 cc. of 5 N aqueous NaOH. After 18 hours the methanol was removed under reduced pressure, 150 cc. of water were added, and the solution was poured into 200 cc. of cold water which contained 30 cc. of 5 N HCl. The granular precipitate which

² Analysis by Mr. William Saschek, Department of Biochemistry, Columbia University, New York.

separated was filtered and dissolved in 150 cc. of acetone. 150 cc. of water were added and the solution was cooled to 0°. The product which crystallized weighed 11.17 gm. and melted at 105–107°. Further dilution yielded a second crop (0.42 gm.), m.p. 103–106°.

The 11.17 gm. were recrystallized from 50 cc. of ether and 50 cc. of benzene at 5°. Dense crystals (11.3 gm.) formed slowly over several days and melted at 105–106° with effervescence.

For analysis a sample was dried at 100° and 0.1 mm. for 24 hours. 11.85 per cent of the weight was lost. $[\alpha]_D = +31^\circ \pm 2^\circ$ ($c = 1$ in methanol).

*Analysis*²— $C_{24}H_{38}O_4$. Calculated, C 73.80, H 9.81; found, C 73.60, H 9.89

Methyl 3(α),12(α)-Dihydroxy-Δ^{9,11}-cholenate (V) (4, 5) and Methyl 3(α),12(β)-Dihydroxy-Δ^{9,11}-cholenate⁴ from Methyl 3(α)-Hydroxy-12-keto-Δ^{9,11}-cholenate (I) (1)—0.404 gm. of I was hydrogenated in 25 cc. of acetic acid and 25 cc. of ethanol (95 per cent) with 2 mg. of PtO₂. In 7.5 hours 0.983 mole of hydrogen had been absorbed and 1.01 moles of hydrogen were utilized after 22 hours. The reduction products were treated as already described.

The molecular extinction coefficient in methanol of methyl 3(α)-hydroxy-12-keto-Δ^{9,11}-cholenate (I) was 11,950. The extinction coefficient of the reduction products was 203. Therefore not more than 1.6 per cent of I could have been present.

The specific rotation of the reduction products was $+47^\circ \pm 2^\circ$ ($c = 1.01$ in methanol). Since the specific rotations of the 12(α) and 12(β) epimeric forms are $+104^\circ$ and $+31^\circ$ respectively, the ratio of the 12(α) to the 12(β) compound appears to be about 1:4.

Methyl 3(α)-Acetoxy-12-keto-Δ^{9,11}-cholenate (VII) (6) from Methyl 3(α)-Hydroxy-12-keto-Δ^{9,11}-cholenate (1)—2.01 gm. of I were acetylated in 2 cc. of acetic anhydride and 2 cc. of pyridine at room temperature for 18 hours. The crystalline mass which separated was dissolved in 30 cc. of benzene and washed with water, with dilute hydrochloric acid, with dilute sodium bicarbonate solution, and with water. The benzene was removed under reduced pressure and the residue was dissolved in 20 cc. of methanol. The first fraction which separated weighed 1.84 gm. and melted at 149.5–150°; the second crop weighed 0.24 gm. and melted at 149.5–150°. The two fractions were combined, dissolved in 20 cc. of boiling methanol, and cooled to 0°. The recrystallized material weighed 1.91 gm. and melted at 150–151°. $[\alpha]_D = +107^\circ \pm 2^\circ$ (30.0 mg. in 3 cc. of chloroform). The molecular extinction coefficient at 240 mμ was 11,600.

⁴ Methyl 3(α), 12(β)-dihydroxy-Δ^{9,11}-cholenate has not been separated in crystalline form. The specific rotation of the ester prepared from the acid VI was $[\alpha]_D = +31^\circ \pm 2^\circ$ ($c = 1.01$ in methanol).

Methyl 3(α)-Acetoxy-12(α)-hydroxy- $\Delta^{9,11}$ -cholenate (VIII) (5, 6) and Methyl 3(α)-Acetoxy-12(β)-hydroxy- $\Delta^{9,11}$ -cholenate (IX) from Methyl 3(α)-Acetoxy-12-keto- $\Delta^{9,11}$ -cholenate (VII) (6)—4.44 gm. of VII were dissolved in 25 cc. of acetic acid and 25 cc. of ethanol in the presence of 30 mg. of PtO_2 . In 27 hours 0.983 mole of hydrogen was absorbed.

The solution was evaporated under reduced pressure, the residue was dissolved in benzene, and the acetic acid was removed with water. The benzene was evaporated and the last traces were displaced with ethyl acetate. The volume was made to 10 cc. with ethyl acetate, 50 cc. of petroleum ether were added, and the solution was cooled to 5°. 3.80 gm. of product which melted at 122–123.5° were separated. $[\alpha]_D = +64^\circ \pm 2^\circ$ (30.7 mg. in 3 cc. of acetone).

After recrystallizing three times from 5 cc. of ethyl acetate and 50 cc. of petroleum ether, the weight was 2.34 gm. and the melting point was 125.5–126°. $[\alpha]_D = +71^\circ \pm 2^\circ$ (31.9 mg. in 3 cc. of acetone).

1 gm. was dissolved in 10 cc. of benzene, diluted with 40 cc. of petroleum ether, and adsorbed on a column of 30 gm. of aluminum oxide. It was eluted with varying proportions of benzene and petroleum ether. The specific rotation of each fraction was taken. The first elutions with specific rotations in acetone which varied from +107° to +98° were combined to give 257 mg. Intermediate fractions with specific rotations varying between +87° and +62° (402 mg.) were discarded. The last fractions (286 mg.), which were removed with 4 per cent methanol in benzene, consisted largely of methyl 3(α)-acetoxy-12(β)-hydroxy- $\Delta^{9,11}$ -cholenate. $[\alpha]_D = +46^\circ \pm 2^\circ$ (106 mg. in 10 cc. of acetone).

This fraction was recrystallized twice from 0.5 cc. of ethyl acetate and 5 cc. of petroleum ether. The weight was 85 mg. and the melting point was 100–101°. $[\alpha]_D = +47^\circ \pm 2^\circ$ (30.4 mg. in 3 cc. of methanol).

*Analysis*⁵— $\text{C}_{27}\text{H}_{46}\text{O}_5$. Calculated, C 72.61, H 9.48; found, C 72.75, H 9.36

The first fraction of 257 mg. which was largely methyl 3(α)-acetoxy-12(α)-hydroxy- $\Delta^{9,11}$ -cholenate was readsorbed on 8 gm. of aluminum oxide and eluted with petroleum ether-benzene, 1:1. 67 mg. thus obtained were crystallized from 0.3 cc. of ethyl acetate and 4 cc. of petroleum ether. 33 mg. of material melted at 135–138°. $[\alpha]_D = +112^\circ \pm 4^\circ$ (15.0 mg. in 3 cc. of methanol).

The combined weight of the next four eluates, each of 50 cc. of 1:1 petroleum ether-benzene, was 89 mg. It was crystallized from 0.3 cc. of ethyl acetate and 4 cc. of petroleum ether. 70 mg. of crystals melted at 105–106°. After drying at 100° and 0.1 mm. for 18 hours the melting point was 116–117°. $[\alpha]_D = +111^\circ \pm 2^\circ$ (30.5 mg. in 3 cc. of methanol).

For identification of methyl 3(α)-acetoxy-12(α)-hydroxy- $\Delta^{9,11}$ -cholenate

⁵ Analysis by Mr. Joseph Alicino, Metuchen, New Jersey.

(VIII), comparison was made with a sample of this compound which had been prepared by treatment of methyl 3(α)-acetoxy-12-chloro- $\Delta^{9,11}$ -cholenate (5, 6) with silver carbonate in aqueous acetone. 500 mg. which were prepared as reported (6) were purified by chromatography on aluminum oxide. Elution was accomplished with benzene and petroleum ether, 1:9, and later with a higher proportion of benzene. A middle fraction which weighed 135 mg. was crystallized from 0.5 cc. of ethyl acetate and 6 cc. of petroleum ether. 123 mg. of the product melted at 96–101°, recrystallized spontaneously on the melting point block, and remelted at 105.5–106.5°. 103.4 mg. of the same sample, when dried at 100° and 0.1 mm. for 6 hours, lost 8.4 mg. and then melted at 116–116.5°. $[\alpha]_D = +111^\circ \pm 2^\circ$ (30.4 mg. in 3 cc. of methanol).

*Analysis*⁴— $C_{27}H_{42}O_6$. Calculated, C 72.61, H 9.48; found, C 72.29, H 9.41

The sample of methyl 3(α)-acetoxy-12(α)-hydroxy- $\Delta^{9,11}$ -cholenate prepared by reduction possessed properties which were identical with those described in the preceding paragraph. When the two samples which had been dried were mixed together, the melting point was not depressed.

However, the melting point is not a satisfactory criterion for identification or for evidence of purity. When equal parts (20 mg. each) of methyl 3(α)-acetoxy-12(α)-hydroxy- $\Delta^{9,11}$ -cholenate (m.p. 116–116.5°) and the epimeric 12(β)-hydroxy compound (m.p. 100–101°) were dissolved in 0.25 cc. of benzene and 3 cc. of petroleum ether added, crystals separated which melted at 120–123°. The specific rotation of the crystals, $[\alpha]_D = +81^\circ \pm 3^\circ$ (20 mg. in 3 cc. of acetone) indicated a mixture of the α and β epimeric forms in the proportion of 53:47. It is interesting to note that the melting point of this mixture is about 7° above that of the α form and about 22° above that of the β epimer. The compound with the β configuration does not separate with solvent in the crystal lattice, but the α epimer may crystallize with solvent.

The molecular extinction coefficient of methyl 3(α)-acetoxy-12-keto- $\Delta^{9,11}$ -cholenate in methanol at 240 $m\mu$ was 11,600, and after reduction the extinction coefficient in methanol was 122. This indicates the presence of less than 1 per cent of unsaturated ketone.

The specific rotation of the mixture of reduction products which was taken before fractional crystallization was $+72^\circ \pm 2^\circ$ ($c = 1.115$ in methanol). Since the specific rotations of the epimeric α and β forms are $+112^\circ$ and $+47^\circ$ respectively, the ratio of the two forms was approximately 2:3.

3(α)-Hydroxy-12(α)-acetoxy- $\Delta^{9,11}$ -cholenic Acid (XII) (4, 5) from 3(α),-12(β)-Dihydroxy- $\Delta^{9,11}$ -cholenic Acid (VI)—780 mg. of VI were dissolved in 78 cc. of acetic acid at room temperature and 2.6 cc. of 5 N aqueous sulfuric

acid were added. After 10 minutes 1.5 gm. of sodium acetate were added, and the solution was concentrated under reduced pressure almost to dryness. The residue was distributed between ether and water and the organic phase was washed with water and concentrated to dryness under reduced pressure. The residue crystallized from absolute ethyl ether to give 640 mg. of the product. After recrystallization from methanol-water 476 mg. melted at 188–189° and did not depress the melting point of 3(α)-hydroxy-12(α)-acetoxy- $\Delta^{9,11}$ -cholenic acid (4). $[\alpha]_D = +206^\circ \pm 2^\circ$ (32.2 mg. in 3.00 cc. of methanol).

Methyl 3(α)-Hydroxy-12(α)-methoxy- $\Delta^{9,11}$ -cholenate (X) (4, 5) from Methyl 3(α),12(β)-Diacetoxy- $\Delta^{9,11}$ -cholenate (IV)—2.44 gm. of IV, 11 cc. of methanol, and 0.20 cc. of 12.0 N HCl were placed in a glass-stoppered flask with 5 gm. of glass beads and rotated for 16 hours. All crystals dissolved. After 3 days at room temperature the solution was cooled to 5° and a mass of crystals separated. After 48 hours 0.910 gm. of product was filtered from solution. Evaporation of the filtrate yielded a second crop of 0.59 gm. The combined crops were suspended in 6 cc. of boiling methanol which contained 50 mg. of sodium acetate and the solution was cooled to 5°. The crystals which separated weighed 1.28 gm. $[\alpha]_D = +131^\circ \pm 2^\circ$ ($c = 1.015$ in chloroform). The melting point (162–162.5°) was not changed when the product was mixed with an authentic sample of X.

Methyl 3(α)-Hydroxy-12(α)-methoxy- $\Delta^{9,11}$ -cholenate (X) (4, 5) from 3(α),12(β)-Dihydroxy- $\Delta^{9,11}$ -cholenic Acid (VI)—3.90 gm. of VI, 22 cc. of methanol, and 0.4 cc. of 12.0 N HCl were allowed to stand at 5° for 3 days. A very small amount of crystals separated. After 1 day at 26° the suspension of crystals which had separated was cooled to 5° for 24 hours and was then filtered. 3.21 gm. of product with a melting point of 161–162° were obtained. Evaporation of the filtrate yielded a second crop of 0.41 gm., which melted at 160–161°. The two crops were suspended in 10 cc. of boiling methanol which contained 50 mg. of sodium acetate. The flask was cooled to 5° and 3.26 gm. of the product were removed by filtration. $[\alpha]_D = +131^\circ \pm 2^\circ$ ($c = 1.006$ in chloroform). The melting point (162–162.5°) was not depressed when the crystals were mixed with an authentic sample of X.

Hydrogenolysis—Hydrogenolysis of the seven compounds listed in Table IV was carried out as follows. The steroid was dissolved in the solvent, the platinum oxide was added, and hydrogenation was carried out for the time indicated. The platinum was removed by filtration, the solvent was evaporated under reduced pressure, the residue was dissolved in benzene, and the last traces of acetic acid were removed with water. The benzene was evaporated under reduced pressure and the residue, if an acid, was converted to a methyl ester with diazomethane. This treatment

was necessary for compound VI. In order to identify the product of hydrogenolysis, the 3(α)-acetyl derivative was prepared with acetic anhydride and pyridine. After removal of acetic anhydride and pyridine by the usual procedure the acetyl compound was separated by crystallization from a mixture of equal parts of acetone and methanol at 5°. In each instance one recrystallization yielded a product with a specific rotation and melting point in agreement with that of an authentic sample of methyl 3(α)-acetoxy- $\Delta^9,^{11}$ -cholenate (XIII) ($[\alpha]_D = +58^\circ$ in acetone; m.p. 137–138° respectively) and did not depress the melting point when mixed with a

TABLE IV
Hydrogenolysis of Substituents at C₁₃

Compound No.		Solvent, acetic acid	Platinum oxide	Time	Hydrogen absorbed	Methyl 3(α)-acetoxy- $\Delta^9,^{11}$ -cholenate (XIII) obtained*
	mm	cc.	gm. per mole	hrs.	moles	mg.
I	0.5	10	100	70	1.99	140
III	0.5	10	100	42	0.94	125
V	0.5	10	100	70	0.96	144
VI	0.5	10	100	41	1.00	128
VII	10.0	†	100	15	1.25	984
X	1.0	‡	150	22	0.98	286
XI	0.5	10	100	25	1.00	142

* The weights of methyl 3(α)-acetoxy- $\Delta^9,^{11}$ -cholenate (XIII) do not indicate the total amount of this compound which had been formed. A quantitative separation was not made and XIII was isolated for purposes of identification only.

† 25 cc. of acetic acid and 25 cc. of ethanol (95 per cent).

‡ 5 cc. of acetic acid and 5 cc. of ethanol (95 per cent).

sample of XIII. The weights of XIII separated in each experiment are given in the last column of Table IV.

SUMMARY

In the presence of Adams' platinum oxide catalyst and hydrogen, the 12-carbonyl group of methyl 3(α)-hydroxy-12-keto- $\Delta^9,^{11}$ -cholenate was almost quantitatively reduced to a mixture of the 12(α)-hydroxyl and 12(β)-hydroxyl groups. Under the conditions used the proportion of these two epimers was 1:4 respectively. Hydrogenation of methyl 3(α)-acetoxy-12-keto- $\Delta^9,^{11}$ -cholenate yielded the corresponding 12(α) and 12(β) epimeric hydroxy compounds in the proportion of 2:3.

With 2 gm. or less of platinum oxide per mole of steroid, utilization of hydrogen stopped after absorption of 1 mole. With larger amounts of

platinum oxide, hydrogenolysis of both 12(α)-hydroxyl and 12(β)-hydroxyl groups occurred with formation of methyl 3(α)-hydroxy- $\Delta^{9,11}$ -cholenate. The 12(α)-acetoxyl and the 12(α)-methoxyl groups were also removed by hydrogenolysis.

Traces of selenium reduced the rate of utilization of hydrogen but did not change the sequence in which the hydrogenation progressed.

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THE CHEMICAL NATURE OF FAT-SOLUBLE MATERIALS WITH BIOTIN ACTIVITY IN HUMAN PLASMA*

ADDITIONAL STUDIES ON LIPIDE STIMULATION OF MICROBIAL GROWTH

By A. E. AXELROD, MILTON MITZ,[†] AND KLAUS HOFMANN

(From the Department of Chemistry, University of Pittsburgh, and the Institute
of Pathology, Western Pennsylvania Hospital, Pittsburgh)

(Received for publication, May 3, 1948)

In a recent paper, Trager (1) described the presence of a material in the plasma of a variety of animal species which on hydrolysis with acids or enzymes yielded a fat-soluble substance possessing biotin-like activity. In a subsequent publication (2) the relationship of this substance to the course of avian malaria was reported. In connection with our search for other forms of biotin in natural materials we had similarly observed the presence in lyophilized human plasma of an ether-soluble, neutral material capable of promoting growth of *Lactobacillus arabinosus* in the absence of biotin (3). Saponification of this material yielded a biologically active saponifiable fraction and a non-saponifiable fraction which was almost devoid of biological activity. This finding is not in agreement with that of Trager (1), who reported that the activity was present in the non-saponifiable fraction. The acidic nature of our active material and the finding of Williams and Fieger (4) that oleic acid possessed biotin-like activity for *Lactobacillus casei* indicated that the activity of our substance might be attributed to its content of known fatty acids. For further characterization the crude acid fraction was esterified and the resulting esters distilled and resaponified. The regenerated acids were subjected to a lead salt-alcohol separation (5) and the activity was found in the liquid fraction. A spectrophotometric analysis (6) of this liquid fraction indicated the presence of approximately 48 per cent of monoethenoid acids (oleic acid), 40 per cent of diethenoid acids (linoleic acid), and 9 per cent of tetraethenoid acids (arachidonic acid). If the activity of the liquid fraction were due to the above components, it is obvious that its biological effects should be duplicated by a mixture prepared from purified oleic, linoleic, and arachidonic acids in the above proportions. Such a synthetic mixture was prepared and its activity was identical with that of the liquid fraction. On the basis of these results there is no need for postulating the existence in

* Supported in part by funds from the American Cancer Society and the Williams-Waterman Fund of the Research Corporation.

[†] Ciba Pharmaceutical Products Junior Research Fellow.

human plasma of unknown ether-soluble, biotin-active substances. All of the activity is explicable in terms of known fatty acids.

In the course of the lead salt-alcohol fractionation, we consistently noted approximately 40 per cent loss in biological activity. Although the liquid fraction represented only about 60 per cent of the total fatty acid fraction, its activity per unit weight was essentially the same as that of the starting material. The solid fraction was practically inactive. This observation led us to investigate the possibility of a synergistic effect between the two fractions. Such an effect was actually observed and explained the above loss in biological activity. Following this, the interesting observation was made that synergistic growth effects were present with a variety of fatty acids. For example, it was noted that palmitic, stearic, and vaccenic acids were also capable of exerting synergistic effects with the liquid fraction. These fatty acids would be expected to accumulate in the solid fraction of a lead salt-alcohol fractionation and could easily explain its synergistic effects. It is significant that none of these acids possessed any appreciable growth-promoting activity when tested alone under our experimental conditions. Synergism between the above fatty acids and oleic, linoleic, and arachidonic acids was also demonstrated. The stimulatory effects of the solid fraction and palmitic, stearic, and vaccenic acids upon the liquid fraction and the synthetic mixtures of oleic, linoleic, and arachidonic acids were identical. This constitutes further support for the concept that the activity of the liquid fraction is due to oleic, linoleic, and arachidonic acids.

In addition, the response of various microorganisms to a variety of lipides has been determined and a preliminary study of the effects of structural modifications of the oleic acid molecule upon its biological activity has been made.

EXPERIMENTAL

I. Microbiological Assay Procedures

Biotin activity was determined by the following procedures: *Lactobacillus arabinosus* (7),¹ *Lactobacillus casei* (8),² *Saccharomyces cerevisiae* (9),³ and the Stoddard (10) strain of *Streptococcus haemolyticus*, group C, mucoid phase (11). For the latter organism, the following modifications of the published procedure (11) were made. The medium was supplemented with the following compounds per liter of double strength medium:

¹ L-Asparagine (0.01 per cent) was added to the basal medium which was adjusted to pH 6.6 before autoclaving.

² An incubation period of 96 hours was employed. The medium with biotin omitted was adjusted to either pH 6.8 or 5.5.

³ The cultures were grown in 50 ml. Erlenmeyer flasks at 30°.

sodium acetate·3H₂O, 40 gm.; sodium citrate·2H₂O, 1 gm.; L-asparagine, 200 mg.; guanine, 20 mg.; xanthine, 20 mg.; pyridoxal hydrochloride, 2 mg.; pyridoxamine dihydrochloride, 2 mg.; p-aminobenzoic acid, 0.1 mg.; *i*-inositol, 1 mg.; pteroylglutamic acid, 1 γ . The medium with biotin omitted was adjusted to pH 6.5 or 5.5 and the cultures grown at 37° for either 24 or 48 hours. 10 ml. of medium were added per culture tube. Periodic neutralization with alkali was not carried out. The variations in pH and incubation time did not affect the extent of growth of the *Streptococcus haemolyticus* in response to the various lipides.

All of the above media are low in biotin and are adequate for growth when supplemented with biotin. For the lactobacilli assays, growth was determined by acidimetric titration with a Beckman pH meter. For the remaining two organisms, growth was estimated turbidimetrically in the Evelyn photocolormeter. The biotin activity of the various lipides was calculated from a standard biotin curve simultaneously determined and expressed as millimicrograms of biotin per mg. of substance.

The lipides were dissolved in redistilled 95 per cent alcohol for assays. Amounts of solution ranging from 0.05 to 0.30 ml. were added per assay tube, and the final volume adjusted to 10 ml. by suitable additions of medium and water. The tolerance of the organisms to alcohol was greater than 0.3 ml. per 10 ml. and this quantity was never exceeded.

II. Isolation of Lipide Fractions from Human Blood Plasma⁴

Extraction—The isolation of the ether-soluble fraction from human blood plasma was carried out essentially according to the procedure of Trager (1). A mixture of 9100 ml. of distilled water, 900 ml. of concentrated sulfuric acid, and 1000 ml. of human blood plasma (prepared from commercial lyophilized blood plasma) was autoclaved at 15 pounds pressure for 1 hour. After cooling to room temperature, the contents were filtered through a 38.5 cm. Reeve Angel No. 820 fluted filter. The filter paper containing the dark precipitate was extracted with three 1000 ml. portions of ether. The combined ether extracts were washed with three 500 ml. portions of 10 per cent sodium bicarbonate solution and three 500 ml. portions of distilled water, and dried over sodium sulfate. The ether was evaporated and the residue dried to constant weight *in vacuo* over phosphorus pentoxide; 5.4 gm. of a dark brown waxy material were obtained (fraction I, biotin activity = 1.6 m γ per mg.). This fraction is comparable to the fat-soluble fraction of Trager.

Saponification—Fraction I (40 gm.) was refluxed under nitrogen for 6 hours with 720 ml. of an alcoholic potassium hydroxide solution (12). After cooling, 1500 ml. of distilled water were added, and separation into

⁴ *Lactobacillus arabinosus* was utilized as the test organism in these studies.

saponifiable and non-saponifiable fractions was made in the usual manner. The non-saponifiable fractions weighed 12.4 gm. (fraction II, biotin activity = <0.15 m γ per mg.), while the saponifiable material weighed 26.0 gm. (fraction III, biotin activity = 5.8 m γ per mg.).

Esterification—Fraction III (26.0 gm.) was esterified with diazomethane in the usual manner, and the resulting methyl esters separated from residues by distillation under nitrogen. An ester fraction boiling from 105 – 110° at 0.005 mm. and weighing 22.0 gm. was obtained (fraction IV, biotin activity = 1.0 m γ per mg.). The distillation residues (biotin activity = <0.15 m γ per mg.) were not investigated further.

Resaponification—Saponification of fraction IV (22.0 gm.) with methanolic potassium hydroxide under nitrogen, and isolation of the resulting free acids in the usual manner, yielded 20.6 gm. of an acid fraction (fraction V, biotin activity = 6.1 m γ per mg.).

Lead Salt-Alcohol Separation—Fraction V was separated into solid and liquid acids by a modification of the Twitchell procedure (5). Fraction V (20 gm.) was dissolved in 200 ml. of hot 95 per cent alcohol and this solution was added to a hot solution of 12 gm. of lead acetate $\cdot 3\text{H}_2\text{O}$ in 200 ml. of alcohol and 2 ml. of glacial acetic acid. The mixture was allowed to stand at 15° for 12 hours. The solid lead soaps were collected on a Büchner funnel and washed with a small quantity of cold 95 per cent alcohol. The soaps were then recrystallized from 400 ml. of 95 per cent alcohol containing 0.5 ml. of glacial acetic acid. The combined filtrates and mother liquors from the solid soaps were evaporated under nitrogen and the liquid acids liberated by warming with 2 N hydrochloric acid and extracted with ether. From the washed and dried ether extracts 13.0 gm. of liquid acids (fraction VI, biotin activity = 6.2 m γ per mg.) were obtained. The solid lead soaps were converted into the free fatty acids as described for the liquid acids, and 5.5 gm. of solid acids were obtained (fraction VII, biotin activity = 0.10 m γ per mg.).

Characterization of Fraction VI—A spectrophotometric analysis (6) of fraction VI (iodine number = 151.4) indicated a composition of 48.4 per cent of monoethenoid acids (oleic acid), 40.3 per cent of diethenoid acids (linoleic acid), and 9.1 per cent of tetraethenoid acids (arachidonic acid). In order to identify the linoleic acid by a crystalline derivative, a 1 gm. sample of the fraction was brominated in ether, and 238 mg. of tetrabromostearic acid (m.p. 114.5 – 116°) were isolated in the usual manner (13).

Analysis— $\text{C}_{18}\text{H}_{32}\text{O}_2\text{Br}_4$. Calculated. C 36.03, H 5.37, Br 53.27
Found. " 35.89, " 5.04, " 53.38

The amounts of ether-insoluble bromides obtained were insufficient for characterization.

III. Preparation of Synthetic Mixture of Oleic, Linoleic, and Arachidonic Acids

A mixture of 480 mg. of oleic acid, 400 mg. of linoleic acid, and 90 mg. of arachidonic acid was dissolved in ether, the ether removed *in vacuo*, and the resulting mixture dried to constant weight *in vacuo*. The mixture had an iodine number of 138.5 (biotin activity = 6.0 m γ per mg.).

TABLE I

Activating Effects of Solid Fraction upon Biotin-Like Activity of Liquid Fraction and Synthetic Mixture

Solid fraction*	Liquid fraction†	Synthetic mixture‡	Biotin activity
γ per tube	γ per tube	γ per tube	m γ per tube
	30		0.20
5	30		0.28
15	30		0.40
30	30		0.44
60 *	30		0.54
100	30		0.55
	50		0.30
75	50		0.65
150	50		0.65
300	50		0.63
		30	0.18
30		30	0.43
60		30	0.52

* Solid fraction from lead salt-alcohol separation of fatty acids from human plasma. This fraction alone possessed no growth-promoting activity in the quantities employed.

† Liquid fraction from the same lead salt-alcohol separation.

‡ Oleic, linoleic, and arachidonic acids (cf. Section III).

IV. Synergistic Growth Effects between Different Fatty Acids⁴

Experiments illustrating the synergistic effects between certain fatty acid fractions obtained from human blood as well as certain purified fatty acids are shown in Tables I and II. The stimulatory effects of the solid fraction obtained from a lead salt-alcohol separation of the fatty acids from human plasma upon the corresponding liquid acids and a mixture of oleic, linoleic, and arachidonic acids are illustrated in Table I. It may be seen that 30 γ of the solid fraction can markedly stimulate the biotin-like activity of 30 γ of both the liquid fraction and the synthetic mixture. It is particularly significant that the degree of stimulation was practically identical in both cases. This finding, in conjunction with the similarity in growth-promoting activity previously described, provides strong support for the concept that

the biotin-like activity of the liquid fraction is due to its content of oleic, linoleic, and arachidonic acids. The degree of stimulation is, within certain limits, a function of the amount of the solid fraction added. Thus maximum stimulation of 30 γ of the liquid fraction is obtained in the presence of 60 γ of the solid fraction.

The results obtained with the complex plasma fractions made it desirable to investigate the synergistic effects between purified fatty acids. A detailed investigation in which varying combinations of fatty acids were employed is summarized in Table II. To simplify the discussion, vaccenic acid and the solid fraction are included among the "saturated" acids.

TABLE II
Synergistic Effects between Various Fatty Acids

The values are expressed as millimicrograms of biotin activity per assay tube.

Unsaturated acids*	No addition	Plus 60 γ per tube of†			
		Vaccenic‡ acid	Palmitic acid	Stearic acid	Solid fraction§
Arachidonic acid	0.05	0.39	0.26	0.10	0.43
Linoleic acid	0.12	0.47	0.35	0.20	0.23
Oleic acid	0.18	0.63	0.29	0.26	0.27
Liquid fraction 	0.20	0.63	0.53	0.31	0.54
Synthetic mixture¶	0.18	0.58	0.56	0.28	0.52

* 30 γ per tube.

† In the concentrations employed, these acids alone were devoid of growth-promoting activity.

‡ Prepared as previously described (14).

§ Solid fraction from the lead salt-alcohol separation of fatty acids from human plasma.

|| Liquid fraction from the same lead salt-alcohol separation.

¶ Oleic, linoleic, and arachidonic acids (*cf.* Section III).

Amounts of the unsaturated acids ranging from 30 to 100 γ per tube were assayed alone and in the presence of varying quantities (5 to 300 γ per tube) of the "saturated" acids. In this manner, both the absolute quantities of unsaturated acids and the relative proportions of "saturated" acid to unsaturated acid were varied. In general, the degree of stimulation was dependent upon the ratio of "saturated" to unsaturated acid. Synergistic effects were first observed at a ratio ("saturated" acid in gm. per unsaturated acid in gm.) of 0.5 and reached a maximum at a ratio of 2.0. The results given in Table II were obtained with this latter proportion. It is apparent that the synergistic effects of the "saturated" acids increased in the following order: stearic, palmitic, and vaccenic acid. Most striking was the stimulating effect of the "saturated" acids upon arachidonic acid,

which itself is almost devoid of biotin activity. Oleic and linoleic acids were stimulated to approximately the same degree by the "saturated" acids. In addition to the "saturated" acids listed in Table II, slight activation of oleic acid with caproic, caprylic, capric, and myristic acids was noted. Lauric acid inhibited the growth activity of oleic acid.

It may be seen from Table II that the stimulatory effects of vaccenic, palmitic, and stearic acids upon the liquid fraction and the synthetic mix-

TABLE III
Biotin-Like Activity of Various Lipides

The values are expressed as milligramms of biotin per mg. of substance.

Lipide	<i>L. arabinosus</i>	<i>L. casei</i>		<i>S. cerevisiae</i>	<i>S. haemolyticus</i>
		pH 6.8*	pH 5.3*		
Oleic acid...	6.0	5.9	11.5	0.60	<0.05
Linoleic acid	4.0	0.58	1.4	<0.05	<0.05
Elaidic "	1.0	10.5	15.2	<0.05	<0.05
Methyl oleatè . . .	3.2	0.75	1.3	<0.05	0.16
Stearic acid	<0.05	<0.05	<0.05	<0.05	<0.05
Tween 80	0.58	1.4	2.9	0.32	0.55
Cholesterol	<0.05	<0.05	<0.05		<0.05
Vaccenic acid	1.2	6.0	5.3	<0.05	
Fraction I†	1.6	3.3	6.6	<0.05	<0.05
" II‡	<0.15	<0.05	<0.05	<0.05	<0.05
" III§	5.8	2.2	10.5	<0.05	<0.05

* pH of the medium before autoclaving.

† Ether-soluble fraction from human blood plasma.

‡ Non-saponifiable portion of fraction I.

§ Saponifiable portion of fraction I.

ture of fatty acids are identical. These effects are similar to those observed with the solid fraction.

V. Biotin-Like Activity of Various Lipides for a Number of Microorganisms

The biotin-like activity of various lipides for *Lactobacillus arabinosus*, *Lactobacillus casei*, *Saccharomyces cerevisiae*, and *Streptococcus haemolyticus* was determined and the results summarized in Table III. It is of interest to note that the organisms fall into two classes with regard to their stimulation by lipide materials. On the media employed, the biotin requirement of the lactobacilli could be satisfied by a number of lipides. In contrast, these lipides were, with but few exceptions, unable to substitute for biotin in the nutrition of *Saccharomyces cerevisiae* and *Streptococcus haemolyticus*.

This variance in response may be due simply to permeability differences. On the other hand, it may denote profound differences in the metabolic events related to biotin.

For *Lactobacillus casei*, the stimulatory activity of the lipides was more pronounced at the lower pH. Williams and Fieger (4) have similarly noted an increased response to oleic acid as the pH was lowered. However, in contrast to these authors, we have observed considerable growth of *Lactobacillus casei* in the presence of oleic acid at pH 6.8.

TABLE IV
Biological Activity of Oleic Acid Derivatives

	Compound	Activity*
I	Oleic acid	6.0
II	Methyl oleate	3.2
III	Oleic acid amide	6.3
IV	Oleyl alcohol	<0.05
V	Linoleic acid	4.0
VI	Linolenic "	1.7
VII	Elaidic acid	1.0
VIII	Vaccenic acid	1.2
IX	Stearic "	<0.05
X	Dihydroxystearic acid (m.p. 94°)	<0.05
XI	" " (" 130.7°)	<0.05
XII	Azelaic acid†	<0.05
XIII	Pelargonic acid	<0.05

* *Lactobacillus arabinosus* was employed as the test organism. Values are expressed as millimicrograms of biotin per mg. of substance.

† Aqueous solution employed for assay.

VI. Biological Activity of Oleic Acid Derivatives

Since oleic acid possesses marked biotin-like activity, it was of interest to ascertain the biological specificity of the molecule. Accordingly, a preliminary study of the effects of structural modifications of the oleic acid molecule upon its biological activity for *Lactobacillus arabinosus* has been made and the results summarized in Table IV. Compounds I to IV illustrate the effects of changes in the carboxyl group of the oleic acid molecule. Whereas the amide (III) was as active as the parent compound, the methyl ester (II) was approximately one-half as active. This difference could be attributed to the greater ease of hydrolysis of the amide linkage as compared to the ester linkage. Replacement of the carboxyl group by a primary alcohol group (oleyl alcohol, IV) resulted in complete loss of biological activity. The results obtained with linoleic and linolenic acids (V and VI) demonstrated that increased unsaturation resulted in decreased activity. The stereochemical specificity of oleic acid was illustrated by the fact that

elaidic acid (VII) was relatively inactive. It is of interest that elaidic acid possesses a high order of activity for *Lactobacillus casei* (Table III). Vaccenic acid (VIII) with a trans double bond in the 11, 12 position had approximately the same activity as elaidic acid. Saturation of the double bond by hydrogenation (stearic acid, IX), as well as by hydroxylation (dihydroxystearic acids, X and XI), led to complete inactivation. From structural considerations, azelaic and pelargonic acids could be considered to function as precursors of oleic acid and were therefore also included in this study. These acids alone or in combination proved to be inactive.

DISCUSSION

The experiments described in this paper have demonstrated that the biotin activity of the fat-soluble fraction from human plasma can be explained in terms of known fatty acids. Of particular interest was the observation that certain saturated fatty acids were capable of exerting pronounced synergistic effects with various unsaturated fatty acids. The ability of the fatty acids to replace biotin in the nutrition of a number of microorganisms under certain experimental conditions provides strong evidence for the function of biotin in fatty acid synthesis (15, 16).

We do not feel that sufficient evidence has been presented by Williams and Fieger (4, 17) to substantiate their view that biotin functions as a factor in cell permeability. A strong argument against their concept is afforded by the observation that numerous analogues of biotin are completely devoid of biotin activity, although, on a structural basis, they would be expected to be even more surface-active than biotin (18). It is conceivable, however, that the synergistic effects herein described may be attributed to surface phenomena.

The rôle of acetate in the synthesis of fatty acids has been established. It should be possible to utilize bacterial systems of the type described in this paper to study the intermediate steps in this synthesis which are, at present, but little understood. For example, the biological activity of possible intermediates in the synthesis of fatty acids could be studied.

The biological activity of the fatty acids for microorganisms suggests their possible activity in replacing biotin for higher animals. MacKay and Barnes have described such a relationship in rats (19). Trager (1), employing the crude ether-soluble, neutral fraction from plasma, reported beneficial effects in biotin-deficient chicks. The effects of purified fatty acids in varying combinations are being investigated in biotin-deficient rats and chicks in this laboratory.

In studies on the metabolism of fatty acids, it would be most desirable to have available accurate micromethods for the determination of individual fatty acids. At the onset of the present investigation, we had hoped

that the growth-stimulating properties of these compounds would serve as the basis for microbiological assay procedures. When the complex inter-relationships between the various fatty acids described in this paper were observed, it became evident that the microbiological assay was unsuitable.

SUMMARY

1. The biotin-like activity for *Lactobacillus arabinosus* of the fat-soluble fraction of human plasma is explainable in terms of its content of known fatty acids.

2. Pronounced synergistic growth effects between various fatty acids have been demonstrated in *Lactobacillus arabinosus*.

3. The response of various microorganisms to a variety of lipides has been determined.

4. Preliminary studies of the effects of structural modifications of the oleic acid molecule upon its biological activity have been conducted.

We wish to express our thanks to Dr. B. F. Daubert for the purified fatty acids and for the spectrophotometric work, to Dr. Erich Baer for the oleyl alcohol, to Dr. Campbell Moses for the lyophilized human plasma, and to the Atlas Powder Company for the Tween 80. We are indebted to Miss Patricia Williams for technical assistance.

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A STUDY OF THE ASSAY METHOD FOR THE GUINEA PIG ANTISTIFFNESS FACTOR*

BY BERT E. CHRISTENSEN, M. B. NAFF, VERNON H. CHELDELIN,
AND ROSALIND WULZEN

(From the Departments of Chemistry and Zoology, Oregon State College, Corvallis)

(Received for publication, April 16, 1948)

In 1941 Wulzen and Bahrs (1) reported a characteristic muscular stiffness at the wrist among guinea pigs on a skim milk diet, which progressed in intensity over a period of months. Autopsies of deficient animals revealed extensive calcification throughout the muscles and internal organs. Raw cream fed by mouth was found to alleviate the wrist stiffness within a few days, although disappearance of calcium phosphate deposits required many months.

A procedure for the isolation of a factor present in raw cream which cured an induced stiffness in guinea pigs was described in 1943 (2). 3 mg. of the factor were isolated from 15 gallons of raw cream. The compound was reported to be an oily ketone (steroid) which was very susceptible to atmospheric oxidation and was active in dosages of 0.1 γ .

The factor present in cream was later traced to the molasses which had been added to the diet of the cow, increasing thereby the original amount in cream. This led to the use of molasses and finally to unheated cane juice as a source of the factor. The procedure for the isolation from molasses or cane juice has been reported (3). 100 mg. of a factor (pure white leaflets, m.p. 81.5–82°) were isolated from 55 gallons of cane juice, which was very susceptible to atmospheric oxidation and was active in dosages of 0.002 γ (3).

Three reviews (4) have pointed out the need for the confirmation of this work by other laboratories, as well as information regarding the reliability and limitations of the assay. Experiments of this nature have been published recently by Oleson and coworkers of the Lederle Laboratories (5). These have confirmed the existence of a stiffness syndrome, as well as the curative power of a crystalline material derived from cane juice, when fed in a 5 γ dosage. Furthermore, several compounds related to the common sterols were reported which exert a similar curative effect.

* This work was supported by the Williams-Waterman Fund of the Research Corporation, New York, and the General Research Council of the Oregon State System of Higher Education, Corvallis. Published with the approval of the Monographs Publication Committee, Research paper No. 123, School of Science, Department of Chemistry.

Since the work on the isolation and identification of the natural factor at Oregon State College was interrupted,¹ experiments were undertaken by the authors to confirm the earlier work and carry the problem to completion. Samples of molecularly distilled cane wax (equivalent when first made to that reported as containing 10,000,000 units of activity per gm. of material (3), curative in 0.1 γ dosage) were obtained for this purpose from Eli Lilly and Company.² However, when this material was separated into various fractions by means of a flowing chromatograph, it was not possible to follow further concentration changes. The results from the assay were frequently contradictory and the precision was so low that further concentrations of the active principle could not be followed with any degree of certainty.

Although the assay has evidently been adequate to establish the presence or absence of the syndrome, additional evidence is required to fix its limitations and to ascertain whether it can serve as an analytical tool for isolation of the natural factor. Since this is a crucial matter, a careful check of the assay procedure was undertaken.

This problem was approached by dilution experiments as well as other treatments. Samples of the wax were prepared in various dilutions. These were assayed and the results clearly indicate no differentiation among animals fed wax over a several fold concentration range. They confirm the Lederle experiments (5) which show that there is no significant difference in animals fed the 1 and 5 γ dosages.

The wax was also subjected to a number of treatments (adsorption, molecular distillation, treatment with pure oxygen at 100° for 24 hours, removal of the ketone fraction, fractional crystallization, etc.) which should yield fractions with varying degrees of potency. These materials, together with a number of other samples (*i.e.* ertron, the Lilly factor, an active sterol (Lederle), and Cuban cane wax)² which for various reasons were thought to possess marked activity, were submitted to assay. The identities of all samples were unknown to the analyst at the time the assays were performed. All the data obtained over a period of 1 year, in which duplicate assays (in groups of three to eight animals) were made, are tabulated in Table I.

If the method has quantitative value, there should be significant differences among the assays of the animals receiving the different treatments.

¹ Due to the resignation of Dr. W. J. van Wagendonk to accept a position at the University of Indiana.

² The authors are indebted to Dr. A. L. Caldwell of Eli Lilly and Company for the samples of cane wax and the highly active component of cane wax, to Dr. A. J. Kunschner of the Nutrition Research Laboratories for samples of ertron, and to Mr. George P. Van Dooren for samples of Cuban sugar-cane wax.

TABLE I
Assays on Materials Used in Treatment of Cane Wax

Group No.	Treatment No.	Material tested	Dosage	Change per animal unit*
			γ	
70		Negative control		-0.1
71		" "		-0.3
75		" "		-0.3
77		" "		0.8
82		" "		0.0
89		" "		0.0
103		" "		0.1
107		" "		-0.1
112†		" "		0.7
157		" "		0.0
163†		" "		1.3
172		" "		0.7
119	1	Lederle sample	100	-0.1
173	1	" "	100	0.8
183	1	" "	100	0.0
174	2	" "	5	0.2
176	2	" "	5	0.1
			mg.	
143	3	FA-5	6	0.0
165	3	" "	6	0.8
161	4	FB-1	6	0.4
155	4	" "	6	0.6
102	5	Wax 2	6	1.2
116	5	" 2	6	1.9
184	5	" 2	6	1.0
74	6	Ketones, Wax 2	1	1.0
90	6	" " 2	1	0.0
134	7	" " 2	6	1.8
139	7	" " 2	6	1.4
162	7	" " 2	6	0.4
168	7	" " 2	6	0.0
135	8	Non-ketones, Wax 2	6	0.0
140	8	" " 2	6	1.0
136	9	Adsorbed, Wax 2	6	0.0
142	9	" " 2	6	0.5
133	10	Unadsorbed, Wax 2	6	0.8
141	10	" " 2	6	0.2
169	11	Fraction FB-6	6	0.0
159	11	" "	6	0.7
76	12	Cuban wax	1	1.3
93	12	" "	1	0.1
78	13	Ertron	1	1.6
94	13	" "	1	1.7
166	14	O ₂ , 24 hrs., 100°†	6	1.0
170	14	" 24 " 100°	6	0.2
171	14	" 24 " 100°	6	1.6

TABLE I—*Concluded*

Group No.	Treatment No.	Material tested	Dosage	Change per animal unit*
			mg.	
175	14	O ₂ , 24 hrs., 100°	6	0.0
B	15	Wax 5	10	0.0
E	15	" 5	10	0.0
G	15	" 5	10	0.0
128	16	" 5	6	2.1
137	16	" 5	6	1.2
147	16	" 5	6	-0.1
A	17	" 5	1	0.0
C	17	" 5	1	0.2
D	17	" 5	1	0.0
F	17	" 5	1	0.5
125	18	" 3A	6	1.2
138	18	" 3A	6	-0.3
148	18	" 3A	6	1.9
I	19	Lilly factor	0.1	0.3
J	19	" "	0.1	-0.3
122	20	Wax 2 (new)	6	-0.2
185	20	" 2 "	6	1.1
157	21	Fraction FB-4	6	1.5
177	21	" "	6	0.2

* The total change with the 1 to 4 system, described previously (3), is given as the sum of the changes in the readings of left and right paws for all animals over a period of 1 week. A change in reading from 3 to 4 in one paw, for example, corresponds to 1 unit.

† Negative controls, Groups 112 and 163, contained 0.2 to 2 mg. of fat acid.

‡ Wax 2.

In order to determine this a statistical analysis³ was made of all the tests in Table I, the negative controls and the animals for which the reading in either paw was missing being excluded. In all, the statistical study deals with twenty-one treatments and 53 groups of animals, consisting of 162 individuals. The results of the analysis are shown in Table II.

Table II gives three sources of variation; namely, (a) the variation among the different treatments, (b) the variation among the groups which received the same treatment, and (c) the variation among the animals which happened to be in the same arbitrary group. The three variances listed in Table II are the respective quantitative measures of these variations. The ratio of the group variance to the animals' variance is given in Table II as 3.40. As the 1 per cent level of Snedecor's *F* distribution, with 32 and 109

³ Statistical treatment of the experimental data was carried out by Dr. Jerome C. R. Li of the Oregon State College Mathematics Department. The authors wish to express their gratitude for his helpful suggestions.

degrees of freedom, is less than 1.89, this variance ratio, 3.40, indicates that the variation among the groups is significantly greater than that among the animals within each group, despite the fact that the animals were arbitrarily divided into different groups.

There is no obvious factor which should cause the various groups to react differently to the same treatment. Some factor not fully controlled such as the incidence of infectious disease in the animal colony or deterioration of the physiological activity of the specimens with time may have contributed to statistical uncertainty. Whatever the reason may be, it illustrates the inadequacy of the method for quantitative assay purposes in its present form.

The variance 1.4302 due to the treatments is approximately equal to the variance 1.3796 due to the groups which receive the same treatment. The variance ratio is 1.04. This clearly indicates that the different treatments

TABLE II
Analysis of Variance

Sources of variation	Degrees of freedom	Sum of squares	Variance	Variance ratio
(a) Treatments	20	28.6030	1.4302	3.40
(b) Groups within treatment	32	44.1461	1.3796	
(c) Animals within group	109	44.2509	0.4060	
Total	161	117.0000		

do not show different effects in this experiment and that it is not possible to differentiate among animals fed samples of materials from cane wax and other sources.

DISCUSSION

Since the data presented here, together with those from the Lederle report, indicate that it is not possible to make comparisons of relative activity with stiffness measurements within a several fold concentration range, it seems doubtful whether any concentrations other than an almost perfect separation, such as removal of wax from cane juice by ether extraction, could be followed. The distribution of active materials resulting from the treatment of cane wax either by chromatography, molecular distillation, or fractional crystallization would hardly be likely to occur in such favorable ratios as to permit differentiation between various fractions by this assay procedure. Considering (a) the fact that Oleson and co-workers have shown a number of common sterols to possess antistiffness activity, (b) the low sensitivity of the assay, and (c) the distribution usually

effected in isolation work, it does not seem strange that all the fractions isolated in this laboratory appeared relatively equal in activity and that differentiation between them is an impossibility.

SUMMARY

The assay procedure (2, 3) for the guinea pig antistiffness factor has been studied with respect to its reproducibility and sensitivity of measurement over a several fold concentration range of active sugar-cane wax. Statistical analyses of the results indicate that there are no significant differences among samples of wax receiving different chemical treatments. It is concluded *that the assay procedure is not suitable for quantitative analytical purposes.*

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THE ISOLATION AND PROPERTIES OF PHOSPHO-GLUCOMUTASE

By VICTOR A. NAJJAR*

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis)

(Received for publication, April 22, 1948)

Phosphoglucumutase, the enzyme that catalyzes the conversion of glucose-1-phosphate to glucose-6-phosphate, has been found in tissue extracts of various animals as well as in yeast extracts (1, 2). It has been shown that the enzyme is specific for the above glucose esters; it is activated by Mg^{++} and cysteine and has a pH activity optimum at 7.5 (2). The reaction was proved to be reversible as shown by the *in vitro* synthesis of glycogen from glucose-6-phosphate through the combined action of phosphoglucumutase and phosphorylase (3). Colowick and Sutherland (4) determined the equilibrium of the reaction with a purified preparation of phosphoglucumutase; they found that 5.5 per cent of 1-ester was present at equilibrium at 30°. The position of the equilibrium was independent of pH.

This report deals with the purification, crystallization, and properties of the enzyme.

Estimation of Enzyme Activity

The activity of the enzyme was measured by the conversion of the acid-labile P of the 1-ester into the acid-stable P of the 6-ester. The details of the method were as follows:

The enzyme solution to be tested was diluted appropriately with cold distilled water immediately before use and kept in an ice bath. Enzymatically prepared glucose-1-phosphate was used as substrate; it was made up in a stock solution containing 2×10^{-2} M glucose-1-phosphate and 6×10^{-3} M Mg^{++} (as $MgSO_4$) and adjusted to pH 7.5. A 0.05 M cysteine solution adjusted to pH 7.5 was prepared fresh for each estimation from a 0.5 M stock solution. 0.1 cc. of the substrate (representing 62 γ of acid-labile P) and 0.2 cc. of the cysteine solution were pipetted into a Klett colorimeter tube and left in a constant temperature water bath at 30° for 2 minutes for temperature equilibration. The reaction was started by adding 0.1 cc. of the freshly prepared enzyme dilution. Two or more dilutions of the same enzyme preparation were used for each test. The reaction proceeds in a linear manner up to the point where about 60 per cent of the

* Senior Fellow, National Research Council.

substrate has been used up. This point should not be exceeded in the test (Fig. 1).

After an incubation of 5 minutes duration, the reaction was stopped by the addition of 1 cc. of 5 *N* H₂SO₄. Water was then added to bring the volume up to 5 cc. and the reaction tubes immersed in boiling water for 3 minutes. The inorganic P formed during acid hydrolysis, which is equivalent to the unchanged 1-ester, was measured by the method of Fiske and Subbarow (5). The acid-stable P represented the amount of glucose-6-phosphate formed; it was calculated from the difference in easily hydrolyzable P before and after incubation. The unit of activity was taken as the number of mg. of acid-stable P formed per cc. of enzyme solution under the conditions

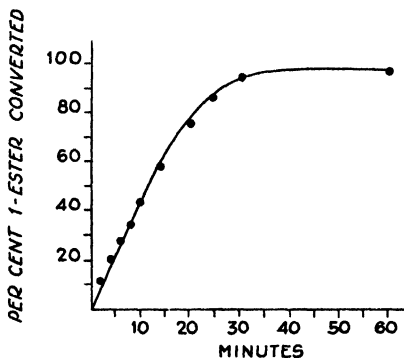


FIG. 1. Time curve of phosphoglucomutase at 30° 4.75 γ of protein per cc., pH 7.5, 0.0025 *M* Mg⁺⁺, and 0.025 *M* cysteine.

described. Protein was estimated by the biuret method of Robinson and Hogden (6).

Method of Purification and Crystallization

Alternate heating and fractionation with ammonium sulfate were the principal procedures used for the purification of the enzyme leading to its crystallization from ammonium sulfate. The following steps are indicative of a typical preparation (Table I). Unless otherwise stated all manipulations were done at 0–4° and pH 5.0.

Two rabbits were given an intravenous injection of nembutal, the vessels of the neck were severed, and the blood allowed to drain thoroughly. The excised rabbit muscles (900 gm.) were minced by passing through a meat grinder. 1 volume (900 cc.) of distilled water was added, stirred thoroughly, and the mixture allowed to stand for 20 minutes, and then strained through gauze. The residue was reextracted with another volume of distilled water.

The combined extracts (1700 cc.), having an activity of 0.5 unit per mg.

of protein, were adjusted to pH 5.0 with 1 M acetic acid. The resulting turbid extract was placed in a water bath at a temperature of 85–90°, and, when the temperature of the extract reached 65°, it was immersed in an ice bath until the temperature reached 4°. Constant mechanical stirring was maintained throughout the heating and cooling procedures. The extract was then filtered through coarse filter paper.

The filtrate (1400 cc.), having an activity of 2.3 units per mg., was brought to 0.65 saturation with solid ammonium sulfate, added gradually with gentle stirring. The precipitate was filtered off or centrifuged and taken up in an equal volume of 0.15 M acetate buffer, pH 5.0. The turbid solution (20 cc.) containing ammonium sulfate at about 0.82 saturation was placed in a water bath at 65° and stirred constantly. The solution was

TABLE I
Sample Protocol for Preparation of Phosphoglucumutase

	Total activity	Total protein	Specific activity
	units	mg.	units per mg.
Water extract (900 gm. muscle)	9850	19,700	0.5
1st heat filtrate (65°)	7800	3,400	2.3
0.0–0.65 ammonium sulfate ppt.	7480	1,160	6.45
2nd heat filtrate (63°)	4370	336	13.0
1st crystallization, 0.50–0.60 ammonium sulfate	2030	112	18.0
2nd crystallization, 0.50–0.60 ammonium sulfate	1080	47	23.0
2nd crystallization, 0.60–0.65 ammonium sulfate	1040	40	26.0

allowed to reach 63° and to remain at that temperature for 3 minutes. The heavy precipitate which formed was centrifuged off and the supernatant fluid (16 cc.) was found to contain 21 mg. of protein per cc. with an activity of 13 units per mg. of protein.

This solution was diluted with 6 volumes of 0.32 saturated ammonium sulfate to obtain a protein concentration of about 3.0 mg. per cc. The ammonium sulfate saturation was then raised to 0.50 by slow, dropwise addition of a saturated solution and the mixture allowed to stand in the cold for 30 minutes. The precipitate was centrifuged off and discarded. The clear supernatant fluid was then warmed to 30° and kept at this temperature for $\frac{1}{2}$ hour. A protein precipitate¹ which formed was centrifuged off, care

¹ This protein had very low mutase activity and a negative temperature coefficient of solubility, and at this ammonium sulfate concentration, it completely dissolved below 10°. It was similar in this respect and in its electrophoretic mobility to rabbit serum albumin.

being taken to prevent cooling. The resulting supernatant fluid was placed in an ice bath and the ammonium sulfate concentration brought up gradually to 0.55 saturation by very slow addition of the saturated salt solution over a period of 1 hour. A faint turbidity appeared, followed by a thin silky shimmer about half an hour later. The material was allowed to stand overnight.

The slow addition of ammonium sulfate was then resumed until the concentration gradually reached 0.60. It was found that appreciable denaturation could be avoided by adding ammonium sulfate dropwise with a pipette, while the solution was stirred at the same time with a glass rod. The solution was left in the cold for 24 hours for maximum crystallization. The crystals were then centrifuged and dissolved in 0.15 M acetate buffer of pH 5 to give a protein concentration of about 2.0 mg. per cc. The activity in the first crystals corresponded to 18 units per mg.

For the second crystallization the ammonium sulfate saturation was raised to 0.50 and the precipitate, if any, discarded. The supernatant fluid was warmed to 30° and any insoluble protein separated by centrifugation at room temperature. The supernatant fluid was cooled to 0–4° and the ammonium sulfate concentration was raised as previously described to 0.60, at which level a slight turbidity began to form, followed by the formation of crystals. After standing overnight, the crystals were separated and gave an activity of 23 units per mg. protein. The concentration of ammonium sulfate in the supernatant fluid was then raised gradually to 0.65, and after standing 24 hours, further crystals formed having an activity of 26 units per mg. of protein. After a third or fourth crystallization the activity remained at 26 units per mg. and the supernatant fluid had the same activity as the crystals.

The procedure described above proved to be reproducible on numerous trials. On a few occasions, however, when blood could not be properly drained, the enzyme preparations proved difficult to purify. In such instances the second heating step often failed to improve purity, since blood proteins, particularly serum albumin, remained as major contaminants.

Perfusion of the muscle (Table II) avoided this difficulty but had the disadvantage that a bulky precipitate formed during both heating steps. The hind leg muscles were perfused rapidly with cold normal saline solution, no more than 15 minutes being allowed for the procedure. The isolation of the enzyme was then carried out as before, except that the small volume of the extract at the second heating step made it necessary to reextract the precipitate with 2 more volumes of acetate buffer. This avoided the loss of the enzyme which was otherwise retained in the meshes of the heat-denatured precipitate. When perfusion was carried out for more than 15 minutes, the first heating step produced denatured protein that was very difficult to centrifuge or filter off.

Properties of Crystalline Phosphoglucomutase

The crystals are long, thin, very fragile plates. Fig. 2 shows a number of fragmented crystals besides some well formed ones. No fructose-6-phosphate was formed in reaction mixtures in which 90 per cent or more of glucose-1-phosphate had been converted; the crystalline phosphoglucomutase is therefore free of phosphohexose isomerase activity. It was also tested with negative results for amylase, phosphorylase, aldolase, D-glycer-

TABLE II
Sample Protocol for Preparation of Phosphoglucomutase from Perfused Rabbit Muscle

	Total activity	Total protein	Enzyme protein	Specific activity
	units	mg.	mg.	units per mg.
Water extract (500 gm. of muscle)	10,400	17,320	400	0.6
1st heat filtrate	5,460	1,760	210	3.1
0.0-0.65 ammonium sulfate ppt.	4,680	742	180	6.3
2nd heat filtrate	3,690	263	142	14.0
1st crystallization, 0.50-0.60 ammonium sulfate	3,380	161	130	21.0
2nd crystallization, 0.50-0.60 ammonium sulfate	988	43	38	23.0
2nd crystallization, 0.60-0.65 ammonium sulfate	1,040	40	40	26.0

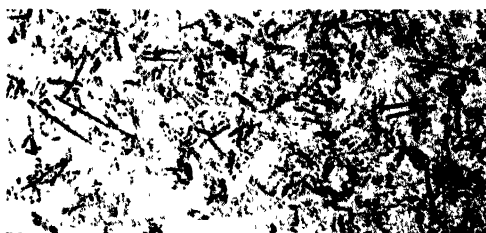


FIG. 2. Phosphoglucomutase crystals from rabbit muscle; $\times 150$

aldehyde phosphate dehydrogenase, glycerophosphate dehydrogenase, triose mutase, enolase, and lactic dehydrogenase.

The absorption spectrum of the enzyme gave a maximum at $278\text{ m}\mu$ (Fig. 3). The extinction of a 1 per cent solution in 1 cm. cell thickness ($E_{1\%}^{1\text{cm.}}$) at $278\text{ m}\mu$ was found to be 7.70.

Electrophoretic studies were made on the crystalline enzyme obtained at 0.65 ammonium sulfate saturation from perfused muscle. The enzyme had an activity of 26 units per mg. of protein. Acetate buffer at pH 5.0 of 0.1 ionic strength was used and the temperature was 1.9° . During 4 hours

a single peak with a mobility of 2.01×10^{-5} (sq. cm. per volt per second) was noted. The protein was positively charged at that pH.

The enzyme was most stable when dissolved in acetate buffer at pH 5.0. Its activity was maintained for about 10 days at ice box temperature, then declined slowly over a period of 6 to 8 weeks. Below pH 4.5 and above pH 8.0 it was readily inactivated.

The enzyme was practically inactive when diluted with water and tested in the absence of cysteine. Maximum activity was obtained when the enzyme was diluted with ice-cold water and tested immediately after dilution in a reaction mixture containing 0.01 to 0.05 M cysteine (Fig. 4). Con-

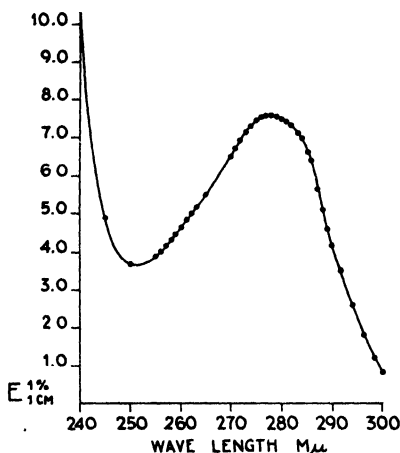


FIG. 3. Absorption spectrum of phosphoglucumutase

siderably lower activity was observed when the enzyme was diluted with cysteine instead of water and then tested in the presence of cysteine.

In Fig. 5 it is shown that in cysteine solution the enzyme lost much more activity, especially during the early period of incubation, than in KCl solution. That such an inactivation by cysteine does not occur while the enzyme is acting on its substrate is shown in Fig. 1; the reaction remains linear until about 60 per cent of the 1-ester is converted to 6-ester. At equilibrium in Fig. 1 the relative amounts of glucose-1-phosphate and glucose-6-phosphate were 5.5 and 94.5 per cent, respectively.

The crystalline enzyme was active without added Mg^{++} , but it was not possible to ascertain whether the enzyme could act in the complete absence of Mg^{++} , inasmuch as traces of the ion present in the preparation or bound to the protein could have been responsible for the observed activity. The optimum range of Mg^{++} concentration, between 0.0005 and 0.0025 M, produced a 4-fold activation (Fig. 6). The decrease in activity with higher

Mg^{++} concentrations is due to an unspecific inhibition of the enzyme by salts (2). In crude muscle extracts, in which mutase was active even when no cysteine was added, Mn^{++} could be used in place of Mg^{++} to increase

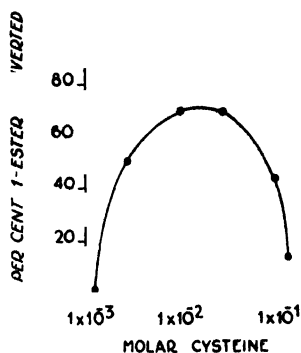


FIG. 4

Fig. 4. The effect of cysteine concentration on the activity of phosphoglucomutase at 30°, pH 7.5, and 0.0025 M Mg^{++} .

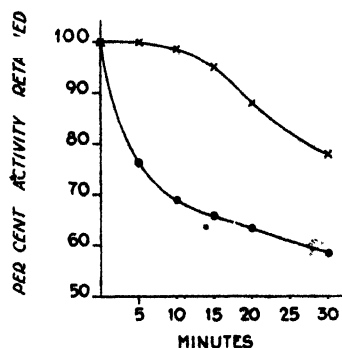


FIG. 5

Fig. 5. Comparison of the effect of incubation of the enzyme in 0.05 M cysteine solution (•) and 0.05 M KCl solution (x) at 30°, pH 7.5. The activity was measured in both cases in the presence of 0.05 M cysteine and 0.0025 M Mg^{++} .

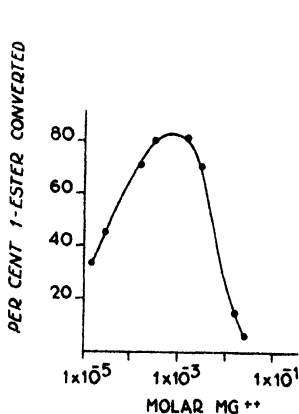


FIG. 6

Fig. 6. The effect of Mg^{++} concentration on the activity of phosphoglucomutase at 30°, pH 7.5, and 0.05 M cysteine.

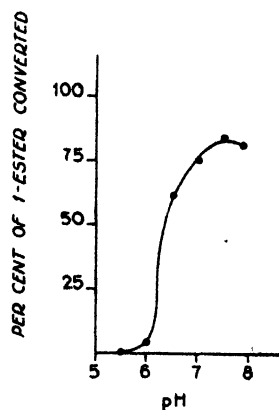


FIG. 7

Fig. 7. The effect of pH on the activity of phosphoglucomutase at 30°, 0.006 M Mg^{++} , and 0.05 M cysteine.

enzyme activity (2). It was not possible to study the effect of Mn^{++} on the purified enzyme, as it forms a complex with cysteine which inhibits enzyme activity. The optimum pH for the enzyme activity was 7.5 (Fig. 7). It

was not possible to test the enzyme at pH values higher than 7.8 because of the formation of magnesium hydroxide.

The turnover number per 100,000 gm. at 30° and pH 7.5 was calculated to be 16,800 moles per minute.

TABLE III

Fluoride Inhibition of Phosphoglucomutase

The reaction mixture contained 1.4 to 1.8 γ phosphoglucomutase, 0.05 M cysteine, pH 7.5, and varying concentrations of the other components shown in the table. All concentrations are expressed in moles per liter.

Variable	Mg	F	Glucose-1-phosphate (initial concentration)	Per cent residual activity Per cent inhibited activity	K*
Mg	5.0×10^{-4}	5.0×10^{-4}	2.2×10^{-3}	5.45	1.50×10^{-12}
"	1.0×10^{-3}	5.0×10^{-4}	2.2×10^{-3}	3.76	2.07×10^{-12}
"	5.0×10^{-4}	1.0×10^{-3}	2.2×10^{-3}	1.42	1.56×10^{-12}
"	1.0×10^{-3}	1.0×10^{-3}	2.2×10^{-3}	0.89	1.96×10^{-12}
"	5.0×10^{-4}	2.0×10^{-3}	2.2×10^{-3}	0.28	1.23×10^{-12}
"	1.0×10^{-3}	2.0×10^{-3}	2.2×10^{-3}	0.30	2.64×10^{-12}
F	5.0×10^{-4}	5.0×10^{-4}	2.2×10^{-3}	5.45	1.50×10^{-12}
"	5.0×10^{-4}	1.0×10^{-3}	2.2×10^{-3}	1.42	1.56×10^{-12}
"	5.0×10^{-4}	1.5×10^{-3}	2.2×10^{-3}	0.71	1.76×10^{-12}
"	5.0×10^{-4}	2.0×10^{-3}	2.2×10^{-3}	0.39	1.72×10^{-12}
"	5.0×10^{-4}	3.0×10^{-3}	2.2×10^{-3}	0.22	2.18×10^{-12}
G-1-P	1.0×10^{-3}	1.0×10^{-3}	1.0×10^{-3}	1.56	1.56×10^{-12}
"	1.0×10^{-3}	1.0×10^{-3}	2.0×10^{-3}	0.77	1.54×10^{-12}
"	1.0×10^{-3}	1.0×10^{-3}	3.0×10^{-3}	0.35	1.05×10^{-12}
Average					1.7×10^{-12}

$$* [\text{Mg}] [\text{F}]^2 [\text{glucose-1-phosphate}] \times \frac{\% \text{ residual activity}}{\% \text{ inhibited activity}} = K.$$

Fluoride Inhibition

Warburg and Christian (7) showed that the inhibition of enolase by fluoride was due to the formation of an inactive magnesium-fluoro-phosphoenolase complex, according to the equation

$$[\text{Mg}][\text{PO}_4][\text{F}]^2 \times \frac{\% \text{ residual activity}}{\% \text{ inhibited activity}} = K = 3.2 \times 10^{-12}$$

The fluoride inhibition of phosphoglucomutase was studied in a similar manner and was found to be dependent on the concentration of the three ions, magnesium, fluoride, and glucose-1-phosphate (Table III). Thus when the concentration of any one ion was varied, and the concentration of the other two ions kept unchanged, a reasonably constant K was obtained with the above equation. The value of the constant obtained under these conditions averaged 1.7×10^{-12} .

It appears that a magnesium-fluoro-phosphate-enzyme complex is formed which is inactive. The phosphate in this complex is the substrate glucose-1-phosphate and probably also its conversion product, glucose-6-phosphate. However, inorganic phosphate also forms an inhibitory complex with magnesium and fluoride, inasmuch as the addition of inorganic phosphate increases the inhibition over and above that exerted by the magnesium-fluoro-1-ester complex.

The concentration of fluoride that gives half maximum inhibition is determined by the concentration of magnesium and 1-ester. With a 1-ester concentration of 2.2×10^{-3} M and a magnesium concentration of 5×10^{-4} M, the concentration of fluoride that gave half maximum inhibition was 1.0×10^{-3} M. With twice the concentration of magnesium and at the same concentration of 1-ester, the fluoride concentration for half maximum inhibition was 0.70×10^{-3} M.

The author wishes to thank Mr. Robert Loeffel for carrying out the electrophoresis study reported in this paper.

SUMMARY

1. A method has been described for the isolation of the enzyme phosphoglucomutase in crystalline form. The enzyme constituted about 2 per cent of the protein extracted from muscle by water and was obtained in the form of thin fragile plates in a yield of about 20 per cent. The activity per mg. of protein was increased about 50-fold in the course of purification, and the product after recrystallization was electrophoretically homogeneous.

2. The enzyme was most stable at pH 5.0 in acetate buffer of 0.1 to 0.3 M, while the optimum for activity was at pH 7.5. For measurement of its activity the enzyme, after dilution with ice-cold water, was added to the reaction mixture containing cysteine. Without cysteine in the reaction mixture the enzyme was practically inactive. Mg^{++} at an optimum concentration of 0.005 to 0.0025 M increased the activity 4-fold.

3. The turnover number per 10^6 gm. of protein per minute at 30° and pH 7.5 corresponded to the conversion of 16,800 moles of glucose-1-phosphate to glucose-6-phosphate.

4. The enzyme was inhibited by fluoride in the absence of inorganic

phosphate. By varying the concentration of the three reactants, it was shown that a complex of $[Mg][F]^2[organic\ phosphate]$ was formed which was presumably competing with magnesium for the enzyme.

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THE EFFECT OF SEVERE HEAT TREATMENT UPON THE AMINO ACIDS OF FRESH AND CURED PORK*

BY J. F. BEUK, F. W. CHORNOCK, AND E. E. RICE

(From the Research Laboratories, Swift and Company, Chicago)

(Received for publication, March 15, 1948)

On the basis of animal experimentation, heat has been shown to have a deleterious effect on proteins (1-13). Some investigators have intimated that dry heat destroys certain amino acids because the addition of these amino acids to the heated protein improves the biological value; others have claimed heat damage to be due to decreased digestibility of the heated proteins.

Fairbanks and Mitchell (1) showed that the addition of lysine to scorched skim milk powder increased the biological value, but that the addition of extra cystine did not. Additions of lysine and histidine, but not cystine, tyrosine, or tryptophan, increased the biological value of the heated casein studied by Greaves, Morgan, and Loveen (2). Similarly, Clandinin *et al.* (3) found that supplementing an overheated soy bean meal with lysine and methionine increased the growth rates of chicks. Hodson and Krueger (4) and Eldred and Rodney (5) reported losses of lysine in heated evaporated milk and heated casein, respectively.

That the digestibility of protein is impaired by dry heat was reported by Chick (6), who found that heating lactalbumin lowered its biological value and significantly decreased its digestibility. Heating casein at a higher temperature decreased both the biological value and the digestibility. Using total protein or sulfur, cystine, or methionine in the undigested protein as criteria, Evans (7) reported raw soy bean meal and meal which had been autoclaved for 30 minutes at 100-130° to be more digestible than meal which had been autoclaved at 130° for 60 minutes as determined either by the Chick method or *in vitro* with trypsin and erepsin. Seegers and Mattill (8) found a marked decrease in the digestibility of the protein of dried liver which had been heated for 72 hours at 120°. However, acid hydrolysates of fresh liver and heated liver gave approximately equal growth rates, biological values, and digestibilities when supplemented by tryptophan. These writers stated: "It is believed that the low biological value . . . is the result of a decreased digestibility such that the resulting amino acid proportions are not representative of the original protein."

Block *et al.* (9) and Zittle and Eldred (10) have shown that raw and

* Presented before the Division of Biological Chemistry of the American Chemical Society at New York, September 17, 1947.

heated casein yielded the same amounts of lysine on acid hydrolysis determined by chemical isolation and by lysine decarboxylase methods, respectively. Riesen *et al.* (11), using microbiological assays of soy bean oil meals for individual amino acids, found that autoclaving 4 hours decreased the amounts of lysine, arginine, and tryptophan which could be liberated by 2 N HCl. Enzymatic digestion, measured in terms of amino acids liberated, improved during proper heat treatment but decreased after prolonged heating.

There have been few investigations of the conditions normally existing in the preparation of edible meats. These have indicated that heating causes slight, possibly insignificant, reductions in amino acid contents. Thus Schweigert *et al.* (12) showed retention of 86 to 106 per cent for leucine, isoleucine, and valine during the curing and cooking of various cuts of meat, and Greenhut *et al.* (13) found the retention of tryptophan and phenylalanine to be 73 to 96 per cent and 91 to 110 per cent, respectively, for several types of meat. No losses of lysine were found by Wilder and Kraybill (14) during the cooking of fresh pork, but a 12 per cent loss was shown for an autoclaved pork luncheon meat.

The small losses always pose the question as to whether amino acids have been destroyed or whether experimental conditions have prevented measurements sufficiently exact to detect the amount of loss. The use of extreme conditions similar to those which have been shown to reduce the biological values of dry proteins offers a means of detecting possible losses and of estimating the significance of losses which may occur in normal processing. This technique has been applied in the present study of the effect of heat upon the amino acid content of fresh and autoclaved pork.

EXPERIMENTAL

Preparation of Samples—A 50 pound lot of lean pork trimmings was comminuted in a high speed chopper ("silent cutter"), mixed several times to assure uniformity, and divided into two portions. One portion was mixed with a standard commercial curing formula;¹ the other remained untreated. Both were held in a cooler at 2–3° for 48 hours. This holding period was necessary to permit normal curing of the meat. The two lots of meat were mixed separately and then packed in 5 ounce cans and sealed under a vacuum in a semiautomatic can closer. Several cans of each portion were stored at –29° as controls. The remaining cans were autoclaved for 24 hours at 112°, cooled immediately with cold water, and stored at –29°.

Preparation of Hydrolysates—Acid hydrolysates of each of the four sam-

¹ This formula contained salt, sugar, nitrate, and nitrite in proportions conforming to the regulations of the United States Department of Agriculture, Bureau of Animal Industry.

ples were prepared by refluxing 10 gm. portions with 100 ml. quantities of 8 N HCl for 8 hours. Previous experiments had indicated these conditions to be adequate for complete liberation of the amino acids and that the treatment destroyed no detectable amount of any amino acid other than tryptophan.

Enzyme digests of the samples were prepared by treating 10 gm. portions suspended in 100 ml. quantities of water with 100 mg. of commercial trypsin powder. The suspensions were adjusted with NaOH to pH 7.5 and incubated for 24 hours at 37°. Then 100 mg. quantities of erepsin were added to the suspensions which, after readjustment to pH 7.5, were incubated for an additional 24 hours. The pH values of the digests were checked periodically during the 48 hour incubation and readjustments were made whenever necessary. Toluene was used as a preservative throughout the incubation periods. At the completion of the trypsin-erepsin treatment, the digests were brought to pH 4.5 and heated in a steam bath to inactivate the enzymes and to remove the toluene.

Acid and enzyme hydrolysates were diluted to 250 ml. volumes, filtered, and stored at 2°. The hydrolysates were neutralized during subsequent dilutions. The amino acid contents of enzymatic digests of raw pork made in this way were generally slightly lower than those of the acid hydrolysates (see the second column in Tables I and II). This may have been due to losses during filtration, since filtered enzyme digests of raw pork contained 5 to 10 per cent less nitrogen (corrected for nitrogen of the enzyme) than did the acid hydrolysates.

Assay Procedures—Except for confirmatory tests with the chemical method for histidine (15), microbiological assay methods were used exclusively in this series of experiments. Leucine, isoleucine, valine, methionine, and tryptophan were determined by use of *Lactobacillus arabinosus* 17-5 with the medium described by Shankman (16). Cystine, methionine, and lysine were determined with *Leuconostoc mesenteroides* P-60 with Medium C of Dunn *et al.* (17), modified by a 5-fold increase of arginine (Kirch (18)). Histidine was determined with *Streptococcus lactis* R and arginine with *Lactobacillus delbrueckii* LD5 with the media described by Stokes *et al.* (19). The media and the manipulative techniques were essentially those reported except that final volumes in the assay tubes were 5 ml. and that 0.05 N sodium hydroxide was used for titrations.

Determination of Effect of Autoclaving—To determine the effect of severe autoclaving, enzyme digests and acid hydrolysates of fresh, autoclaved, fresh cured, and autoclaved cured samples were prepared. All solutions were assayed simultaneously for each amino acid. Check analyses made several months later confirmed the initial values. Average values and retentions are listed in Tables I and II. For simplicity, the values are given

per gm. of sample, this being a valid basis since there was no opportunity for change in weight during autoclaving.

DISCUSSION

Analysis of the acid hydrolysates of the autoclaved samples indicated almost 100 per cent retention of the amino acids except cystine. Cystine showed a 56 per cent retention in both fresh and cured samples after autoclaving (Table I). A sulfide odor from the autoclaved samples indicated the possible breakdown of a sulfur-containing amino acid.

Since tryptophan is acid-labile, analyses for that amino acid were not

TABLE I
Acid Hydrolysis; Retention of Amino Acids in Fresh and Cured Pork after Autoclaving at 112° for 24 Hours

	Fresh pork			Cured pork		
	Raw	Autoclaved	Retained	Raw	Autoclaved	Retained
	mg. per gm.	mg. per gm.	per cent	mg. per gm.	mg. per gm.	per cent
Threonine	9.0	9.0	100	8.7	8.7	100
Leucine	13.5	13.5	100	13.3	13.3	100
Isoleucine	9.0	9.3	103	8.9	9.1	102
Valine	9.5	9.7	102	9.5	9.6	101
Methionine	3.8	3.9	102	3.7	3.7	100
Phenylalanine	6.5	6.6	101	6.6	6.8	103
Cystine	2.7	1.5	56	2.7	1.5	56
Lysine	14.0	13.2	95	13.4	12.8	96
Arginine	7.8	7.5	96	7.5	7.4	99
Histidine	6.5	6.6	101	6.5	6.0	93
Tryptophan*	1.90	1.90	100			

* Alkaline hydrolysis (possibly slight destruction during refluxing (20)).

made on the acid hydrolysates. Assays of alkaline hydrolysates (5 N NaOH for 8 hours, under a reflux) gave similar values for fresh and autoclaved samples. The 1.9 mg. of tryptophan found per gm. of sample correspond to 1.1 per cent tryptophan in the protein, a value slightly lower than the 1.4 per cent reported by Greenhut *et al.* (20). Apparently, partial destruction of tryptophan occurred during refluxing, an occurrence which would be expected in view of the report of the latter authors that alkali partially destroys and racemizes the tryptophan. However, if there were equal rates of loss during hydrolysis of the raw and autoclaved samples, the 100 per cent retention value for the autoclaved sample should be valid.

The apparent amino acid retention based on enzyme hydrolysates varied from 27 per cent for cystine to 56 per cent for leucine in the autoclaved

fresh sample and from 33 per cent for cystine to 65 per cent for isoleucine in the autoclaved cured samples (Table II). This finding is in accord with the marked reduction in the biological values² of these raw and cured pork samples during autoclaving, as found by Lockhart.³ Loss of cystine was not in itself sufficient cause for the decreases in biological value, as indicated by failure of cystine to improve markedly the rate of gain when added to the diet in place of a small amount of the protein. Replacement of autoclaved protein with raw protein permitted rapid gain. The low apparent retention cannot be due to the destruction of the amino acids during autoclaving, since the acid hydrolysates showed a definite loss of cystine only. It may be caused by a decrease in the digestibility of the protein in the autoclaved

TABLE II
Enzyme Hydrolysis; Retention of Amino Acids in Fresh and Cured Pork after Autoclaving at 112° for 24 Hours

	Fresh pork			Cured pork		
	Raw	Autoclaved	Retained	Raw	Autoclaved	Retained
	mg. per gm.	mg. per gm.	per cent	mg. per gm.	mg. per gm.	per cent
Threonine.....	8.4	3.4	41	8.0	2.8	35
Leucine.....	13.1	7.4	56	11.6	6.6	57
Isoleucine.....	8.7	4.3	49	7.6	4.9	65
Valine.....	9.3	5.0	54	9.3	5.4	58
Methionine.....	3.9	1.8	48	3.7	1.8	49
Phenylalanine..	6.8	3.2	47	6.5	3.4	54
Cystine.....	2.6	0.7	27	2.1	0.7	33
Lysine.....	13.7	6.4	47	13.0	5.0	39
Arginine.....	7.4	3.8	52	7.0	3.8	56
Histidine.....	5.3	2.8	53	4.6	2.9	63
Tryptophan....	1.7	0.6	35	1.6	0.6	38

samples. The curing mixture does not seem to affect the retention of the amino acids whether measured on acid or enzyme hydrolysates.

Enzyme digestion of raw samples yielded clear solutions in which small amounts of finely divided material were suspended. Similar treatment of autoclaved meat resulted in turbid solutions containing a larger quantity of insoluble particles. Micro-Kjeldahl analyses showed precipitates from enzyme-digested raw meat to contain 3 to 5 per cent of the nitrogen of the sample; those from autoclaved samples contained 9 to 16 per cent. Hence, part of the low apparent retention is due to the removal of insoluble material during filtration of the digests of cooked samples. This, however,

² Rat growth comparisons.

³ Unpublished data, Lockhart, H. B., Research Laboratories, Swift and Company.

cannot account for the entire apparent loss, since calculation of the values for the filtrates on a nitrogen basis did not greatly improve recovery values. Furthermore, the nitrogen content of the precipitates removed from enzyme hydrolysates of the cooked samples amounted to only 9 to 16 per cent of the total nitrogen, but the lowest apparent amino acid loss was 35 per cent. This would indicate the presence in the filtrates of some amino acids in a form not available to the bacteria used in making the assays.

To determine whether the amino acids in the enzyme digests could be recovered by acid hydrolysis, unfiltered enzyme digests of the four samples were treated with 8 N HCl for 8 hours as described under "Preparation of

TABLE III
Enzyme Hydrolysis Followed by Acid Hydrolysis; Retention of Amino Acids in Fresh and Cured Pork after Autoclaving at 112° for 24 Hours

	Fresh pork			Cured pork		
	Raw	Autoclaved	Retained	Raw	Autoclaved	Retained
	mg. per gm.	mg. per gm.	per cent	mg. per gm.	mg. per gm.	per cent
Threonine	8.1	8.1	100	7.7	7.5	98
Leucine	13.6	14.8	109	13.1	12.9	99
Isoleucine	9.3	9.9	106	8.1	8.5	105
Valine	9.6	9.5	99	9.7	9.6	99
Methionine	3.7	3.9	105	3.6	3.6	100
Phenylalanine	6.3	6.5	103	5.3	5.1	97
Cystine	2.6	1.4	54	2.3	1.3	57
Lysine	14.5	14.5	100	14.5	12.8	90
Arginine	7.7	7.4	96	6.9	7.0	101
Histidine	6.3	6.8	108	6.6	6.5	99

hydrolysates." This procedure did not appreciably affect the values for raw samples, but it did increase the amino acid levels found for the autoclaved samples to such an extent that no marked losses were indicated except for cystine (Table III). These values agree with those in Table I within the range of experimental error. Furthermore, preliminary analyses of acid-hydrolyzed filtrates of enzyme-digested, cooked samples indicated increases in the quantities of amino acids available to the test organisms. Definite increases in total free amino acids measured by the method of Folin, as modified by Sahyun (21), are obtained under such circumstances.

Since the amino acids, except cystine, survive autoclaving for 24 hours at 112°, it is to be expected that the much less severe household and commercial processes would likewise cause no losses other than possibly that of cystine. Whether or not reductions in biological values or in enzyme di-

gestibility would occur under the milder conditions of normal cooking schedules remains to be determined.

SUMMARY

Determinations of the effects of severe heat treatment upon the amino acids of fresh and cured pork were made by comparing the amino acid contents of the raw and cured pork with those of samples which had been autoclaved at 112° for 24 hours.

Analyses of acid- or alkali-hydrolyzed autoclaved samples indicated complete retention of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The retention of cystine was 56 per cent.

Low apparent retention (27 to 65 per cent) was observed when the autoclaved samples were hydrolyzed with enzymes. However, treatment of these enzyme hydrolysates with acid permitted recovery of the initial amounts of all of the amino acids except cystine. The low apparent retention was probably due to the failure of the enzymes to liberate completely all of the amino acids of the autoclaved pork. Part of the bound amino acid fraction is soluble but unavailable both to bacteria and to rats.

The failure of severely treated pork to show losses of amino acids other than cystine makes it seem improbable that ordinary cooking or processing operations destroy any significant amounts of amino acids except cystine. Decreases in the enzyme digestibility suggest the possibility that the milder processes of home or commercial cooking may likewise decrease digestibility, but that the changes may not be detectable under normal conditions.

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INCORPORATION IN VITRO OF RADIOACTIVE CARBON FROM CARBOXYL-LABELED DL-ALANINE AND GLYCINE INTO PROTEINS OF NORMAL AND MALIGNANT RAT LIVERS*

By PAUL C. ZAMECNIK, IVAN D. FRANTZ, JR., ROBERT B. LOFTFIELD,
AND MARY L. STEPHENSON

(From the Medical Laboratories of the Collis P. Huntington Memorial Hospital of Harvard University, at the Massachusetts General Hospital, Boston, and the Laboratory of Nuclear Science and Engineering, Massachusetts Institute of Technology, Cambridge)

(Received for publication, April 14, 1948)

The greater growth rate of a malignant tissue as compared with its tissue of origin implies an increased net rate of protein synthesis (total synthesis minus degradation). It was demonstrated recently (1) that C^{14} -labeled alanine can be incorporated into proteins of liver slices by incubation of the slices in a Krebs-Ringer-phosphate (2) solution containing the alanine. The present work applies this technique to the question of whether or not a malignant tissue *in vitro* synthesizes peptide bonds more rapidly than does its normal counterpart. Use of the tissue slice rather than the whole animal was chosen in order to study protein synthesis under conditions more readily within experimental control than those in the living animal.

Materials and Methods

Young male rats of the Harvard colony¹ were placed for 4 months on a diet containing *p*-dimethylaminoazobenzene (3). At the end of this time they were returned to a normal ration.² 2 months thereafter, exploratory laparotomies were performed. Note was made of those animals bearing small, isolated hepatomas. During the next month, individual rats were killed by decapitation, and the livers were quickly removed. Hepatoma nodules approximately 1 cm. in diameter were carefully dissected free from adjacent liver. Such nodules were firm, pearly white, and free from gross

* Presented in summary at the meeting of the American Association for Cancer Research, Atlantic City, March 12, 1948.

This work was supported in part by grants-in-aid from the American Cancer Society (recommended by the Committee on Growth of the National Research Council) and the Godfrey M. Hyams Fund, and in part by contracts of the Office of Naval Research with Harvard University and with the Massachusetts Institute of Technology.

This is Reprint No. 647 of the Cancer Commission of Harvard University.

¹ These rats were obtained originally from Sprague-Dawley stock. The strain has been kept in Hisaw's laboratory during the past 15 years, with brother and sister matings being done over several generations periodically.

² Pratt's Nurishmix.

evidence of necrosis. In several experiments, larger nodules were used, and here the peripheral, firm portion, approximately 5 mm. in thickness, was selected for slicing. Sections 0.5 mm. in thickness were cut by means of a Stadie-Riggs (4) slicer. Similar slices were cut from grossly non-malignant portions of the same liver and from the livers of control rats of the same strain and sex.

We were early made aware of the rather frequent great variability of one slice from another in its capacity to incorporate alanine into its proteins. Difference in pressure exerted on each section during the cutting, in cell trauma inflicted by the knife, and in the proximity of the section itself to the surface of the liver or to the larger portal vessels may offer partial explanation for this unpredictable behavior of sections grossly similar. As a routine practice, blocks of tissue of the order of $8 \times 6 \times 5$ mm. in size were cut, and then sectioned on the slicer. The top and bottom slices were regularly discarded.

Pieces from areas adjacent to those used were also routinely saved for histological examination. The tumors were principally parenchymal cell hepatomas, type II (5). In a number of tumors there were also smaller areas consisting of adenocarcinoma, hepatoma type I, and bile duct proliferation. In some cases, peritoneal and diaphragmatic metastases provided additional evidence of malignancy. The grossly non-malignant portions of the hepatoma-containing liver uniformly showed evidence of diffuse nodular hyperplasia and cirrhosis. The primary hepatoma was chosen for the present study *because it provided opportunity for comparison of the malignant tumor in each case directly with its tissue of origin in the same animal.*

Into Warburg flasks were pipetted 1.0 cc. of Krebs-Ringer-phosphate solution and 0.1 cc. of a solution containing 0.44 mg. of C¹⁴-carboxyl-labeled DL-alanine (6). The total radioactivity per flask was approximately 10,000 counts per minute, as measured by solid counting. The pH of these two constituents was 7.40. Two slices of control liver or three slices of hepatoma were usually added to a single flask, because the amount of protein in two slices of control liver was found to be approximately equal to that in three slices of hepatoma. 0.2 cc. of 10 per cent potassium hydroxide was placed in each center well. The flasks were incubated, with shaking, in 100 per cent oxygen at 37.5° for 3½ hours. Manometer readings taken at 15 minute intervals showed a relatively linear oxygen consumption throughout the experiment.

At the end of the incubation, the slices were removed and rinsed twice with 20 cc. portions of water. They were then placed in a test-tube, with 5 cc. of a solution containing 50 mg. of inert DL-alanine, and were homogenized. Next, 5 cc. of 20 per cent trichloroacetic acid were added, and

the precipitated proteins were treated as previously described (1). Briefly, they were washed and hydrolyzed, and the ninhydrin procedure (7) was used to split off the α -carboxyl groups of amino acids present in the hydrolysate (plus the γ -carboxyl of aspartic acid). The carbon dioxide thus liberated was trapped in barium hydroxide, and the amount of barium carbonate formed was determined by titration of the uncombined excess of barium hydroxide with 0.249 *N* hydrochloric acid.

Under our experimental conditions, we have found 0.2 to 0.3 mm to be the maximum amount of carbon dioxide one can evolve in the ninhydrin procedure without the risk occasionally of driving contents of the ninhydrin vessel over into the barium hydroxide during the boiling process.

All samples reported in this paper were counted as barium carbonate, with a commercially available³ end window counter said to have a mica window of thickness 1 mg. per sq. cm. After titration of the barium hydroxide unneutralized by carbon dioxide from the ninhydrin reaction, the barium carbonate was filtered onto No. 42 filter paper and mounted on brass disks, in essentially the manner described by Henriques, Kistiakowsky, Margnetti, and Schneider (8). Before removal from the filtration apparatus, the samples were washed with water and acetone. Air was then drawn through for a few minutes. Weighing of the solid samples of BaCO_3 was found to be unnecessary, because the weights could be calculated with sufficient accuracy from the titrations. We established this fact by filtering some samples in fritted glass funnels, drying, and weighing. The weights agreed with the formula BaCO_3 for the precipitate. Further evidence for this composition was provided by the fact that the theoretical yield was obtained when sulfuric acid was added to the precipitate and the CO_2 measured in a gas burette.

Because sufficient material was not available from the slices to provide a layer of barium carbonate of "infinite thickness," corrections for self-absorption were necessary. We corrected all counts to the values that would have been obtained with 0.1 mm thickness (5.4 mg. per sq. cm., with our apparatus). This point of reference was chosen so that the correction factors would differ little from unity. All counts are expressed in this way unless stated otherwise.

Correction factors covering the range of sample sizes encountered in this investigation, calculated from our experimental data, are plotted in Fig. 1. Our data are in fair agreement with the curves presented by Yankwich, Norris, and Huston (10). Five points calculated from their Table I are included in Fig. 1.

For measurement of the activity incorporated into the slices, only 1024 counts were collected in most cases. With the less active samples, which

³ Radiation Counter Laboratories, Chicago, Illinois.

gave net counting rates about equal to the background (15 counts per minute), the probable error due to statistical fluctuations was therefore about 5 per cent.

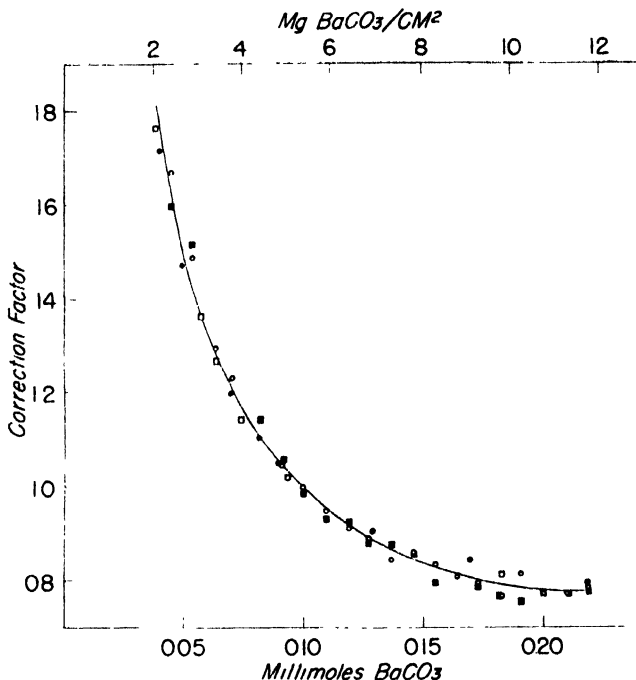


FIG. 1 Correction factors for converting the number of counts per minute observed to the number that would have been observed if exactly 0.1 mm of barium carbonate of the same specific activity had been counted. O, obtained from samples of BaCO₃ of varying weights and constant specific activity; ■, from samples of varying weights and constant total activity; □, calculated from Table I of the paper by Yankwich, Norris, and Huston (10); ●, calculated from the approximate theoretical equation presented by Henriques, Kistiakowsky, Margnetti, and Schneider (8): $I/I_0 = (1 - e^{-\alpha d})/(2\alpha d)$. The mass absorption coefficient, α , is assumed to be 0.28 sq. cm. per mg. for C¹⁴. Solomon, Gould, and Anfinsen (9) quote the value 0.285 ± 0.008 from a personal communication from Yankwich. All samples were prepared by addition of a slight excess of a solution of BaCl₂ to a solution of Na₂C¹⁴O₃ and filtration in the usual manner. The weights were calculated from the molarity of the Na₂CO₃, which was checked manometrically with the Van Slyke manometric gas apparatus and gravimetrically by filtration of BaCO₃ precipitates in fritted glass funnels.

Table I illustrates the over-all reproducibility of the method, including the decarboxylation, titration, transfer, filtration, mounting, and counting, with material of activity high enough to reduce statistical fluctuations below the other errors.

Table II presents similar data from aliquots of a tagged hydrolysate of comparatively low activity.

Results

The principal results of the experiments are listed in Table III, which indicates that *the rate of incorporation of C¹⁴ from labeled alanine into the*

TABLE I
Over-All Reproducibility, with Samples of High Specific Activity

Net counts per min.	Deviation from mean
	<i>per cent</i>
1127	3.6
1049	3.6
1107	1.7
1064	1.5
1103	1.0
1079	1.0

Each sample was prepared by decarboxylation of 0.1 mm of labeled DL-alanine.

TABLE II
Over-All Reproducibility, with Samples of Low Specific Activity

mm BaCO ₃	Net counts per min.	Deviation from mean
		<i>per cent</i>
0.126	30.0	6.1
0.126	31.9	0.4
0.127	33.3	3.7
0.127	34.2	6.4
0.126	30.7	3.6
0.127	30.2	6.1
0.191	37.2	4.7
0.167	34.9	1.0
0.066	22.9	9.4

These samples were prepared by decarboxylation of aliquots of a tagged protein hydrolysate.

proteins of the hepatoma slices is consistently greater than it is into slices of normal control liver and into the grossly non-malignant part of the hepatoma-containing livers. It is clear that the difference in counts per minute per 0.1 mm of barium carbonate obtained in duplicate vessels was in some cases large. As mentioned previously, this is interpreted as a reflection of some unknown biological difference between the slices themselves rather than an indication of some artifact introduced in processing the finished slice (cf. Tables I and II for reproducibility of recovery and counting procedures).

The number of millimoles of barium carbonate present in the samples is of course a measure of the total amount of protein present in the slice.

Separations of Amino Acids—The question arises as to whether all of the C¹⁴ detected in the protein hydrolysates resides in the carboxyl group

TABLE III

Incorporation of Radioactivity Derived from Alanine into Proteins of Normal Liver and Hepatoma

Experiment No.	Normal control liver		Control part of hepatoma-containing liver*		Hepatoma	
	mm BaCO ₃	Counts per min.	mm BaCO ₃	Counts per min.	mm BaCO ₃	Counts per min.
1	0.125	44	0.125	88	0.140	279
	0.110	49	0.125	78	0.116	326
2	0.203†	31	0.099	85	0.083	230
	0.125†	30	0.099	84	0.096	194
3	0.109	85	0.102	111	0.098	394
	0.087	61	0.096	112	0.090	478
	0.093	46				
	0.112	40				
4	0.176	27			0.075	193
	0.202	28			0.112	199
5	0.149	30	0.139	39	0.146	95
	0.208	23	0.160	36	0.166	109
6	0.263	17	0.199	131	0.187	169
	0.263	24	0.186	116	0.131	283
			0.241	73	0.166	253
			0.191	81	0.161	268
7	0.174	36	0.174	94	0.127	329
	0.131	57	0.138	145	0.139	275
8	0.189	21				
	0.200	25				
9	0.108	30				
	0.113	32				
Average..	0.157	37	0.148	91	0.127	255

In each experiment, duplicate vessels were set up for each type of liver slice. Results from each of the vessels are recorded. In a single experiment, frequently all three types of livers were used.

* The non-malignant part of a liver which contains hepatoma nodules.

† Pregnant female rat. All other animals are adult males.

of alanine, or whether some or even all of it is present in other amino acids. Upon the answer to this question rests the validity of this method of approach to the problem of protein synthesis. In particular, the advisability of using carboxyl-labeled compounds is at stake. Besides the greater ease

of preparation, use of such compounds has the advantage that the ninhydrin procedure, specific for amino acids, is available for recovery of the radioactive carbon. Radioactive carbon dioxide thus appears in the samples for counting with specific activity several times that of material obtained by combustion. Unless the possibility of appreciable transfer of activity to other amino acids can be eliminated, great difficulty in the interpretation of results arises, and for many purposes the advantages mentioned are outweighed. To follow this line of reasoning along, it is likely that pyruvate is formed to some degree as a result of deamination of D- and L-alanine. The carboxyl-labeled pyruvate may then be further degraded to acetate and labeled carbon dioxide, or incorporated into the tricarboxylic acid cycle. Radioactivity originating in bicarbonate or pyruvate might conceivably appear in carboxyl groups of both aspartic and glutamic acids (*cf.* (11)). This possibility is rendered less likely because carbon dioxide is trapped in the center well in the present experiments and therefore is less available for incorporation into these two amino acids. There is the further possibility that the carboxyl group of amino acids other than alanine and the above two might become labeled by some mechanism as yet unknown.

For the solution of the problem at hand, a method for total, quantitative separation of the amino acids in the hydrolysates would be desirable. Lacking such a method, we obtained fairly complete separations of four amino acids by adding a large amount of the amino acid in question to the tagged hydrolysate and recrystallizing. The procedure was as follows: Ten Warburg flasks containing three hepatoma slices and a medium containing radioactive alanine were incubated for 3½ hours in oxygen. The slices were pooled and homogenized, and the proteins were prepared and hydrolyzed, with the usual precautions to prevent contamination with tagged alanine from the suspending medium. Excess hydrochloric acid was removed by distillation *in vacuo* at a temperature below 40°. The pH was adjusted to 6.8 by addition of ammonia. The volume was adjusted to 10 cc., three 1 cc. aliquots were decarboxylated, and the activity measured. To four other 1 cc. aliquots were added, respectively, DL-alanine 150 mg., glycine 150 mg., L-glutamic acid 50 mg., and DL-aspartic acid 50 mg. To each of the last three flasks were added 2.5 mg. of DL-alanine in order to reduce by dilution possible contamination of the desired amino acid with tagged alanine. Each solution was brought to the boiling point, and 3 cc. of absolute ethanol were added slowly with heating. The flasks were set aside for 24 to 48 hours, and the crystals were filtered off. The crystals were redissolved in water, more alanine was added to the last three flasks, and the procedure was repeated. For the second crystallization, the volume of solvents and the amount of alanine added were reduced to allow for loss in the first crystallization, so that the concentrations remained the

same. A third crystallization was done in the same way. The crystals were dried, and aliquots of each amino acid were decarboxylated for counting. The number of counts per minute found was multiplied by the factor

$$\frac{\text{Mg. amino acid added}}{\text{Mg. amino acid decarboxylated} \times \text{c.p.m. per cc. hydrolysate}}$$

to give the fraction of the activity in the hydrolysate accounted for by the amino acid under consideration.

TABLE IV

Test of Effectiveness of Separation Procedure. Results of Crystallizations of Amino Acids from Untagged Hepatoma Protein Hydrolysate to Which Was Added Radioactive DL-Alanine Following Hydrolysis

Sample No.	Amino acid	CO ₂ from aliquot	Net counts per min. per 0.1 mm	Counts per min. in total amount of amino acid added*	Per cent activity in hydrolysate accounted for
		mm			
1	Glutamic	0.101	2.0	6.8	0.4
2	"	0.103	3.3	11.2	0.7
3	Aspartic	0.097	3.1	23	1.5
4	"	0.124	2.7	20	1.3
5	Glycine	0.108	1.6	32	2.1
6	"	0.093	0.8	16	1.0
7	Alanine	0.116	85.6	1440	92
8	"	0.106	90.9	1530	98
9	"	0.136	91.1	1533	98
10	"	0.130	88.5	1490	95
11	"	0.127	91.0	1530	98
12	"	0.129	84.9	1490	95
13	"	0.129	88.5	1562	100

Conditions used have been described in the text. Triplicate ninhydrin determinations and counts were performed on 1 cc. aliquots of hydrolysates, with the following reproducibility: 0.102 mm, 1543 c.p.m.; 0.100 mm, 1567 c.p.m.; 0.102 mm, 1580 c.p.m. Counts are expressed as net counts per minute in 1 cc. of hydrolysate, if counted at a thickness of 0.1 mm of barium carbonate. Since all of the activity in the hydrolysate is known to be present as alanine, the percentages reported in the last column for Samples 1 to 6 necessarily represent contamination, and the deviations from 100 per cent in Samples 7 to 13 are due to experimental error.

* Corrected to 0.1 mm thickness.

To test the effectiveness of the method of separation, a similar experiment was carried out with a hydrolysate of untagged hepatoma proteins, to which tagged alanine was added after hydrolysis. The results of these experiments are presented in Tables IV and V.

Within the limitations of accuracy of the experiments, it appears that

practically all of the activity in the protein is incorporated as alanine. Incorporation of a few per cent as other amino acids still cannot be ruled out, but we have obtained no positive evidence for it.

Rate of Incorporation of Activity—The percentage of alanine present in the liver proteins was determined by a colorimetric method (12). The

TABLE V

Results of Crystallizations of Amino Acids from Protein Hydrolysate Prepared from Slices of Hepatoma Which Had Been Incubated for 3½ Hours with Radioactive DL-Alanine

Sample No	Amino acid	CO ₂ from aliquot	Net counts per min. per 0.1 mm	Counts per min. in total amount of amino acid added*	Per cent activity in hydrolysate accounted for
		mm			
1	Glutamic	0.113	2.2	7.5	3.5 ± 0.7
2	"	0.083	2.4	8.2	3.8 ± 0.8
3	Aspartic	0.111	0.6	4.5	2.1 ± 1.5
4	"	0.115	0.4	3.0	1.4 ± 1.5
5	Glycine	0.126	0.4	8	3.7 ± 3.8
6	"	0.139	0.3	6	2.8 ± 3.8
7	Alanine	0.126	12.0	202	94 ± 4.2
8	"	0.149	13.0	218	102 ± 4.3
9	"	0.138	12.9	217	101 ± 4.7
10	"	0.135	14.1	246	115 ± 4.8
11	"	0.138	11.9	200	94 ± 4.7
12	"	0.137	11.7	197	92 ± 4.7

The conditions used are described in the text. Triplicate ninhydrin determinations and counts were performed on 1 cc. aliquots of hydrolysate: 0.076 mm, 220 c.p.m.; 0.077 mm, 216 c.p.m.; 0.080 mm, 205 c.p.m. (Counts are expressed as net counts per minute in 1 cc. of hydrolysate if counted at a thickness of 0.1 mm of barium carbonate.) Because of the relatively low specific activity of the hydrolysate in comparison with that described in Table IV, statistical fluctuations in counting caused appreciable error in this experiment. 1280 counts, divided into four runs of 320 counts each, were collected from each sample. Background counts were made between individual runs. The probable errors shown refer to error due to statistical fluctuations in counting only. Backgrounds averaged 15.4 c.p.m.

* Corrected to 0.1 mm thickness.

results obtained on separate normal livers and hepatoma nodules are listed in Table VI.

From these results it is possible to calculate the percentage of radioactivity taken up by the liver slice and the rate of incorporation of alanine into the slice. 10,000 c.p.m., contained in 5 micromoles of DL-alanine, were added to each vessel. 5000 c.p.m. were thus present in 2.5 micromoles of

L-alanine. An average of 62 c.p.m. was incorporated into the proteins of the slices in a single vessel. This latter figure represents 1.2 per cent of the total counts added as L-alanine. An average of 0.16 mm of amino acids was found after hydrolysis of the protein present in the two liver slices contained in a single vessel. If 8 per cent of the total moles of amino acids is L-alanine, then there are 13 micromoles of L-alanine present in the slices in a single vessel. Finally, the 62 c.p.m. incorporated into the proteins have been accounted for almost completely in the alanine fraction of the protein hydrolysate. These 62 c.p.m. represent 0.03 micromole of L-alanine, or 0.24 per cent of the total alanine in the slices. In summary, approximately 0.2 per cent of the total protein-bound alanine in the slices appears to have been derived from alanine molecules originating in the incubation medium. These rough calculations do not take into account the possibility that

TABLE VI
Molar Fraction of Liver Amino Acids Accounted for As Alanine

Normal livers	Hepatoma nodules
0.095	0.092
0.063	0.083
0.089	0.087

On aliquots of the same protein hydrolysates, ninhydrin and colorimetric alanine determinations were carried out. The moles of carbon dioxide liberated during the ninhydrin procedure (minus the carbon dioxide liberated from the γ -carboxyl group of aspartic acid) represent the number of moles of amino acids present in the aliquot used. The colorimetric alanine determination gives a value for the alanine fraction of these moles of amino acid.

D-alanine may be acted on by D-amino acid oxidase to yield pyruvic acid, and then converted to L-alanine by transamination.

Experiments with Labeled Glycine—It may be stated on the basis of the foregoing experiments that the rate of uptake of alanine into the proteins of hepatoma slices is greater than it is into the proteins of normal liver slices. The question now arises whether this difference is peculiar to alanine metabolism or whether it may hold for other amino acids as well. With this question in mind the following experiment was performed. Slices were made from three types of hepatic tissues. To the incubating medium of certain of the slices was added, as usual, carboxyl-labeled C¹⁴-DL-alanine, and to others carboxyl-labeled C¹⁴-glycine. The result of this experiment, shown in Table VII, indicates that radioactivity derived from glycine is incorporated more rapidly into the slice proteins of the hepatoma than it is into the normal liver. This result is therefore qualitatively in accord with

that for alanine. Since equimolar concentrations of DL-alanine and glycine, containing the same concentrations of radioactivity, were used, it is suggestive that the rate of uptake of radioactivity from glycine into normal liver protein is more rapid than it is from alanine. No accounting was made, however, of the activity present in the various amino acids of the slice proteins when labeled glycine was added. It is thus possible that the labeled carbon derived from glycine may have turned up in the slice protein in amino acids other than glycine.

TABLE VII

Comparison of Rates of Incorporation into Liver Proteins of C^{14} Activity Derived from Alanine and Glycine

Type of liver tissue	Type of labeled amino acid	Counts per min.
Control	DL-Alanine	36
"	"	57
Control-hepatoma*	"	94
"	"	145
Hepatoma	"	329
"	"	275
Control...	Glycine	180
"	"	138
Control-hepatoma.....	"	270
"	"	338
Hepatoma	"	505
"	"	563

The same two rats furnished hepatic tissues for both types of experiment. 10,000 c.p.m., contained in 0.44 mg. of DL-alanine, were added to the appropriate vessels, and 10,000 c.p.m., contained in 0.37 mg. of glycine, were similarly added. While the same number of moles of DL-alanine and glycine was used, the L-alanine represented only half the concentration of the glycine. A strict comparison of the rates of uptake would require the use of L-alanine, free from the presence of the D form. One cannot multiply the alanine values given by 2 because of the possibility of conversion of labeled D-alanine to L-alanine via deamination and transamination.

* Control part of hepatoma-containing liver.

Miscellaneous Related Experiments—An experiment was performed on the rate of incorporation of C^{14} -alanine into the proteins of fetal livers as compared with various other types. The results are shown in Table VIII. In this experiment it is clear that the fetal liver slices incorporated alanine into their proteins at a rate approaching that of the hepatoma. In this connection, Friedberg, Schulman, and Greenberg (13) very recently expressed the view that homogenates of fetal liver synthesize protein more rapidly than homogenates of adult liver. Similarly, liver slices removed 48 hours after partial hepatectomy incorporated alanine into their proteins at 3 times

the control rate.⁴ An accelerated rate of peptide bond synthesis is evidently not a unique property of the hepatoma, but does serve to distinguish its protein metabolism from that of resting adult liver tissue.

The possibility was considered that the hepatoma and fetal liver might contain extractable substances capable of stimulating protein synthesis in slices of normal liver. A well known phenomenon observed in tissue culture work is the growth stimulation an explant of cells derives from the addition to the medium of an aqueous extract of an embryo. With this consideration in mind, various tissue extracts, concentrated and dilute, were added to the medium in which control liver slices were incubated. As indicated in Table IX, however, the extracts, including the rat embryo extract, had no stimulating effect on the incorporation of alanine into the

TABLE VIII
Uptake of Activity from C¹⁴-Alanine into Fetal Liver

Type of liver	Counts per min.
Fetal*	177
	180
Hepatoma	230
	194
Control-hepatoma	84
	84
Normal liver	31
	30

* 15 day-old rat fetuses.

slice proteins. In fact, the addition of a concentrated homogenate of normal liver to the incubation medium depressed the uptake of alanine into the slice proteins.

An experiment was carried out to answer the question whether the thickness of the slice was an important consideration as related to the uptake of activity. With the Stadie-Riggs slicer, if pressure is exerted on the lucite piece which covers the tissue block, and if the knife blade is pressed firmly against the upper edge of the slot through which it is moved, a thinner slice may be obtained than is otherwise produced. It was from this point of view conceivable that some artifact might be introduced due to the difference in cutting properties of the normal liver and hepatoma. It appears from the data in Table X, however, that such variations in thickness of the slice as might occur would not critically influence the uptake of radioactivity.

⁴ Frantz, I. D., Jr., and Bucher, N. L., unpublished data.

The possibility was considered that addition to the incubation medium of a substance similar in structure to alanine might interfere with the uptake of alanine. Accordingly, benzoylalanine was added. It had, however, no clear cut effect on the incorporation of alanine into either normal liver or hepatoma.

The rate of oxygen consumption of the slices was followed for two reasons: (1) to provide evidence that the slice was surviving during the time of

TABLE IX "
Effect of Addition of Tissue Extracts on Uptake of Radioactivity into Liver Slice Proteins

Experiment No.		Counts per min.
1	Control	31
		30
	" + concentrated embryo homogenate	27
		30
	" + " control "	9
2		6
	"	46
		40
	" + control liver extract	50
		57
	" + hepatoma extract	40
		54
	" + fetal liver extract	59
		41

The data are expressed as previously described. The concentrated homogenates were made as follows: 2 gm. of 15 day-old rat embryo were minced and homogenized in 8 cc. of Krebs-Ringer-phosphate buffer. The pH was adjusted to 7.4. 2 gm. of control liver were similarly prepared. Slices of control liver were then incubated in 1 cc. of each of these media.

The extracts were prepared as follows: 0.4 gm. of the appropriate type of liver tissue was minced and ground in a mortar in 10 cc. of Krebs-Ringer-phosphate solution. The suspensions were centrifuged, and 0.2 cc. of the supernatant was added to 0.8 cc. of Krebs-Ringer-phosphate solution. Slices were incubated in this medium. 0.1 cc. of DL-alanine was added in the usual way.

incubation and (2) to find out whether there was a correlation between the rate of oxygen consumption and the rate of protein synthesis. The data summarized in Table XI imply that such a correlation does exist. The hepatoma slices incorporate more alanine (*i.e.*, appear to synthesize more protein) per microliter of oxygen consumed than do the normal liver slices.

In a single experiment, C^{14} -alanine was injected subcutaneously into a rat bearing a small hepatoma. 2 hours later, viviperfusion (15) was carried

TABLE X

Effect of Thickness of Slice on Rate of Uptake of Alanine into Liver Proteins

		mm BaCO ₃	Counts per min.
Normal control liver	Thin	0.149	30
	"	0.208	23
	Thick	0.224	24
	"	0.249	24
Hepatoma	Thin	0.146	95
	"	0.166	109
	Thick	0.259	104
	"	0.191	192
Control-hepatoma	Thin	0.139	39
	"	0.160	36
	Thick	0.264	25
	"	0.253	34

TABLE XI

Relation between Oxygen Consumption and Uptake of Radioactivity

	O ₂ consumed	Counts per min. incorporated	Counts per min. incorporated per micro- liter O ₂ consumed
	<i>microliters</i>		
Control.....	50 ± 7	37 ± 10	0.74 ± 0.19
Control-hepatoma.....	56 ± 6	91 ± 19	1.6 ± 0.23
Hepatoma.....	81 ± 8*	255 ± 62*	3.1 ± 0.74*

At least seven values are included in each arithmetic mean given above. Each mean is followed by the probable error, computed from the spread of the data (not from the number of counts accumulated). It should be pointed out that these are probable errors, not probable errors of the mean, and are indicative of the degree of variation from experiment to experiment rather than of the degree of accuracy to which the mean is known.

Since it was undesirable to dry the slices at the end of the incubation period prior to hydrolysis of the proteins, the oxygen consumption is not expressed as Q_{O_2} . The oxygen consumption is related to the quantity of barium carbonate obtained by ninhydrin decarboxylation of the amino acids present in the hydrolysate of the slice proteins. The values for oxygen consumption and counts per min. incorporated are therefore expressed per 0.1 mm of barium carbonate, which is a way of relating these data to the quantity of protein present in the tissue slice.

* Signifies that the difference from control and control-hepatoma is significant ($P < 0.01$) (14).

out, the animal's blood being washed out by a large volume of Tyrode's solution. The liver was then removed, and the hepatoma nodule was dissected free from surrounding non-malignant liver tissue. A 50 per cent

greater rate of incorporation of labeled alanine into the hepatoma proteins was found than into the non-malignant liver proteins.

DISCUSSION

It is probable that a liver slice, separated from its normal nutritional supply by a traumatic process, and immersed in an extracellular type of medium, does not provide optimal conditions for protein synthesis. The net over-all process in this situation is doubtless proteolysis. Since less than 1 per cent of the alanine molecules contained in protein has been incorporated during the time of incubation, *a difference in the rates of the protein-degrading processes, no matter how great, in the normal liver and hepatoma, cannot account for the higher percentage of radioactivity in the hepatoma.* The greater rate of uptake of labeled amino acid into proteins of the hepatoma thus implies a greater rate of peptide bond synthesis. This conclusion is at variance with that which Rittenberg and Shemin (16) reached as a result of studies of incorporation of stable isotopes into whole animals.

While it appears reasonable to conclude from the present experiments that alanine is incorporated into proteins (and trichloroacetic acid-precipitable peptides) of liver slices, this finding may be interpreted in at least two ways. First, alanine may be built into protein or peptide molecules as they are being newly formed. Secondly, alanine from the incubation medium may be exchanged for alanine molecules present in the interior of existing protein molecules. Both processes may conceivably take place. Since the present experiments do not permit a decision between these possibilities, the term "exchange" has been avoided.

Finally, the present experiments suggest the possibility that the slice technique for measuring the rate of protein synthesis in normal liver and hepatoma may be used as a screen test for potential tumor inhibitors. If an agent can be found capable of inhibiting selectively protein synthesis in hepatoma slices, it is worthy of more extensive trial on whole animal tumors.

SUMMARY

The rate of incorporation of C^{14} -carboxyl-labeled DL-alanine into the proteins of rat liver slices was investigated. The rate of uptake of activity in surviving slices of hepatoma nodules was 7 times that of slices of normal livers and $2\frac{1}{2}$ times that of slices from the non-malignant portion of the hepatoma-containing livers. A preponderance of the activity incorporated was accounted for in the alanine fraction of the proteins. An increased rate of uptake of activity into hepatomas was likewise found when C^{14} -carboxyl-labeled glycine was used. It is concluded that, under the conditions used, the primary *p*-dimethylaminoazobenzene-induced rat hepatoma incorpo-

rates alanine more rapidly into protein than does normal resting adult rat liver.

The authors wish to thank Miss Ann Werner and Mrs. Ruth Slaiman for technical assistance. They are indebted to Professor Joseph C. Aub and Professor Robley D. Evans for valuable advice and encouragement.

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THE QUANTITATIVE SEPARATION OF PURINES, PYRIMIDINES, AND NUCLEOSIDES BY PAPER CHROMATOGRAPHY

By ROLLIN D. HOTCHKISS

(From the Hospital of The Rockefeller Institute for Medical Research, New York)

(Received for publication, March 10, 1948)

The separation of amino acid mixtures by migration with organic solvents in filter paper has been successfully accomplished by many workers since it was first described by Consden, Gordon, and Martin (1). Each amino acid travels in a more or less well defined spot in the body of uniformly migrating solvent and can be visualized in the dried paper as a local spot giving a color reaction with ninhydrin. The present paper reports the separation of the purines and pyrimidines contained in nucleic acids, and several related compounds, by the movement of a boundary of *n*-butyl alcohol along paper strips. Vischer and Chargaff (2) have described the principal steps of a procedure for separating the two bases, guanine and adenine, from nucleic acid in a moving body of a quinoline-collidine mixture. These purines were located in the paper by precipitation of mercury sulfide after formation of the insoluble mercury salts and washing in dilute acid. After complete removal of the quinoline and collidine it was possible to identify the guanine and adenine by their absorption maxima in the ultraviolet range.

Advantageous features of the method described below are as follows: (a) The five bases, cytosine, thymine, uracil, adenine, and guanine, can in microgram quantities be completely separated from one another. (b) The separated substances are detected by ultraviolet spectrophotometric examination of solutions prepared by soaking excised portions of the paper in water. (c) An organic solvent is used which does not interfere with ultraviolet spectrophotometry. (d) Recovery of the individual constituents is essentially quantitative, with an error of 10 per cent or less. (e) Spectrophotometric standards of purity are provided which make it possible to identify each component and (f) to determine with considerable accuracy the composition of binary mixtures which may result from a migration not carried far enough to give altogether complete separations. (g) 1 mg. or less of a nucleic acid can be examined for the rate of liberation of its nitrogenous bases during partial enzymatic or acid hydrolysis. (h) Some simple derivatives such as nucleosides can be purified and identified in this procedure. (i) Less soluble substances, or dilute solutions, are used by evaporation of moderately large volumes of solution upon a small spot in the paper, locally heated.

Certain aspects of the method remain unsatisfactory or disadvantageous: (a) Guanine does not migrate appreciably in butyl alcohol and so it remains with unhydrolyzed nucleic acid, nucleotides, or other alcohol-insoluble substances in the initial spot. Without use of a second solvent, guanine can therefore be isolated in pure form only from certain simple mixtures or completely hydrolyzed material. (b) Phenylalanine (and possibly tryptophan, but not tyrosine) may, if present, appear on the paper in the region occupied by cytosine. Its presence can be recognized through the ninhydrin color reaction or through spectrophotometric study of the fractions. While certain synthetic phenols and aromatic acids would likewise interfere, probably very few natural products will be found to do so. (c) As in other paper chromatography, large quantities of the substances or even of certain foreign materials often tend to make the separation less sharp. (d) Variations in water content of the paper, or other ill defined factors, make it difficult to predict exactly the position that will be occupied by isolated compounds; relative positions and relative rates of flow, on the other hand, are constantly reproducible.

Some of the disadvantages mentioned may eventually be overcome through use of successive solvents or a different initial solvent. The method as described promises to be useful in the quantitative study of the composition of nucleic acids, their constituents and derivatives, and the manner of their enzymatic or hydrolytic degradation.

EXPERIMENTAL

Apparatus—The majority of the chromatograms were developed in a Pyrex glass cylinder surmounted by a flanged aluminum ring closely fitting the cylinder and serving as the support for brass arms holding horizontally placed cylindrical glass troughs and glass rod separators, about 140 mm. in length. A glass plate is placed upon the plane upper surface of the aluminum ring to give a closed system. Early experiments were successfully carried out in an ordinary glass and metal aquarium resting upon one end and closed at the side with a glass plate. In this chamber a wood and metal ring stand supported a rectangular, glass staining dish cover, which served as the trough. The metal used was not observed to cause any disturbances in either of the chambers; wood, however, becomes swollen and saturated with solvent vapor, and should not be used in systems with more than one organic solvent.

Volumes of solution larger than 0.017 cc. were placed upon a small area of the paper, locally heated by curving the strip over a horizontal glass tube, 10 mm. in diameter, joined between a flask of boiling water and a reflux condenser. The fluid samples are delivered from a calibrated capillary pipette (3) by touching it to the paper from time to time as drying occurs.

There has been no indication that the presumptive local drying of the paper has influenced the resolution of either pyrimidines, purines, or amino acids.

Solutions were examined in 3 cc. quantities in the model DU Beckman photoelectric quartz spectrophotometer.

Materials—The paper used was Whatman No. 1, as recommended for amino acid determinations (1). Aqueous extracts of this paper have only very low absorption in the ultraviolet, as discussed below. *n*-Butanol was used as the organic solvent in all of the work reported here. The bottom of the chamber was covered with equal portions of butanol and water and the aqueous phase was brought to about 2.5 per cent concentration of gaseous ammonia by addition of concentrated ammonia. The troughs contained butanol saturated with water at the prevailing room temperature, without added ammonia.

Two lots of each of the principal bases were used: from Hoffmann-La Roche, Inc. (Basel), adenine, guanine, and uracil, kindly made available by Dr. A. Claude; from the Schwarz Laboratories (New York), adenine sulfate, guanine, thymine, and yeast nucleic acid; from the Eastman Kodak Company (Rochester), uracil and yeast nucleic acid; from the Dougherty Chemicals (Richmond Hill, New York), cytosine. In addition, crystalline samples of thymine, cytosine, xanthine, hypoxanthine, adenosine, cytidine, guanosine, and thymidine, prepared in the laboratory of the late Dr. P. A. Levene, were employed. Desoxyribonucleic acid was prepared from calf thymus according to the method of Mirsky (4).

Weighed amounts of the pure bases were dissolved in water, neutralized to about pH 7 if necessary, and the concentration accurately determined by Kjeldahl determination of nitrogen. From these solutions the standard curves for pure bases were obtained and the mixtures submitted to separation were prepared. The nucleosides were not available in sufficient quantity for nitrogen analysis; accordingly the relative absorption data are more dependable than the absolute for these substances.

All calculations based upon absolute weight throughout this paper refer to the anhydrous free bases.

✓ Nucleic acids were hydrolyzed in aqueous hydrochloric acid for 2 hours at 120° in the autoclave. The acid was later removed by evaporation to dryness *in vacuo*, and the hydrolysate, dissolved in a small volume of water, was neutralized with sodium or ammonium hydroxide.

Preparation of Paper Chromatogram—The solutions to be investigated are deposited upon spots or bands distributed along a pencilled "starting line" drawn transversely about 50 mm. from one end of the paper strip. The spots are narrow (10 mm. or less) in the direction of flow, but along the starting line are spread for a distance such that each mm. of length carries approximately 1 γ (and preferably not more than 5 γ) of the individual

bases. Most of the work described here was done on paper strips 125×460 mm., bearing three or four initial spots approximately 10×20 to 25 mm. in size, arranged at least 10 mm. apart. The solutions are applied in the manner indicated above to these spots outlined in pencil. Such spots can carry 20 to 100 γ of individual bases or the degradation products of 1 mg. of nucleic acid.

The end of the paper strip is inserted into a glass trough and weighted by a large glass rod so that the starting line, which is near this end, is just at the point where the paper curves over the glass rod separators and projects downward. After the strips are in place and the bottom of the chamber is provided with butanol-water as mentioned, *n*-butanol saturated with water at room temperature is added to the trough and the chamber is closed. A jacket of corrugated paper is placed about the cylinder to protect it from draughts and exposure to radiators or windows, which can have disastrous effects on an unprotected system.

After 16 to 24 hours at about 25° , the position of the butyl alcohol front is marked, and the strip is removed and hung upside down to dry. The individual "lanes" can be cut apart at this time for separate treatment with ninhydrin, etc., or for separate cutting of strip segments.

Transverse segments are now cut from each lane on a trimming board or with scissors at successive chosen intervals, such as every 10 mm. The precision of the method may often justify making segments as narrow as 5 mm., and in vacant areas segments of 20 or 30 mm. width are convenient. Each excised piece is placed in a clean test-tube and then soaked 1 hour or more in 3.5 cc. of distilled water. In this work the paper may be touched with the hands, but is preferably subjected to a minimum of such exposure.

The absorption of the eluted solutions at $260 m\mu$ is determined in the spectrophotometer. A graph or table of this absorption correlated with the distance travelled from the starting line (to the mid-point of the segment) reveals a series of absorption peaks corresponding to the constituents which have been separated from the original mixture. Figs. 1 and 2 and Table I present typical data. It will be seen that the presence of several substances in each mixture is clearly indicated.

The identity of the separated substances is ascertained by more detailed spectrophotometric examination of the various eluted solutions. In the work to be described below, 3 cc. of these solutions were placed in the quartz cuvettes and examined as desired, either in this approximately neutral state or after being made 0.1 *N* in alkali or acid. For the latter purposes, 0.06 cc. (in routine work, 1 drop) of 5 *N* sodium hydroxide was added, followed when desired by 0.05 cc. (or 1 drop) of 12 *N* (or concentrated) hydrochloric acid.

In what follows the procedures are grouped according to four aims to be achieved: (a) rigorous test of the method, or of new solvents, etc., by study

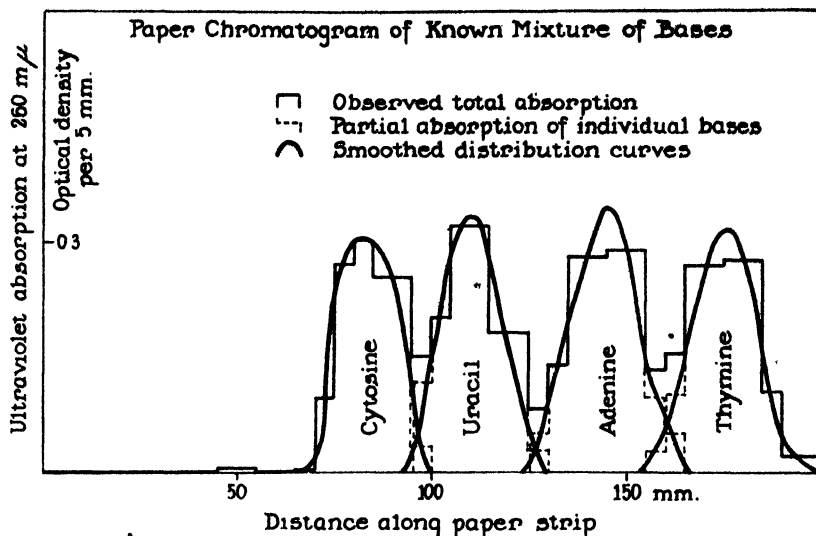


FIG. 1. Distribution of known bases in paper chromatogram

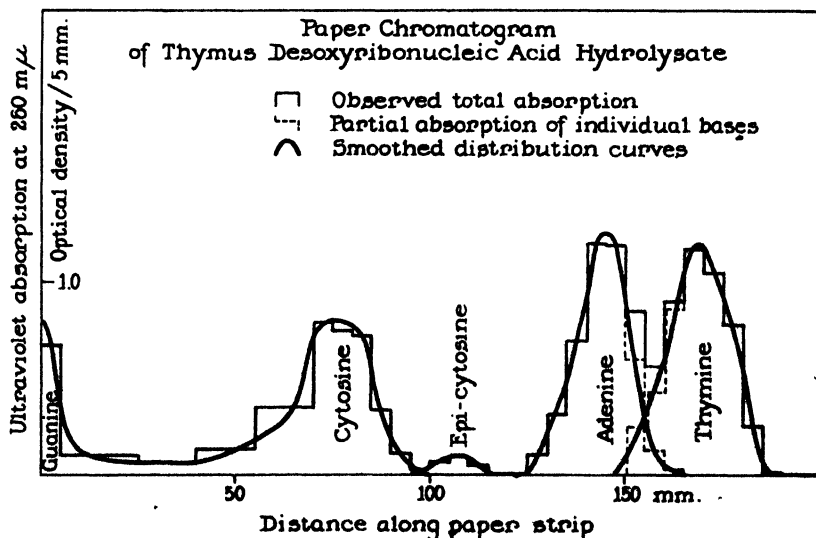


FIG. 2. Distribution of bases from thymus desoxyribonucleic acid hydrolyzed 2 hours at 120° in 2.4 N hydrochloric acid.

of identity and purity of solutions obtained at or near the positions of peak absorption, (b) routine identification of substances isolated near the peaks, (c) routine procedure for determination of quantitative composition of

those pure solutions or binary mixtures which may be obtained at the low points between the absorption peaks, and (d) complete quantitative analysis of mixtures of bases.

Identification of Isolated Major Constituents—Those eluted solutions having high absorption values at $260\text{ m}\mu$ may be further studied to determine their composition. In order to demonstrate in this investigation that pure pyrimidines and purines may be recovered from hydrolysates of nucleic

TABLE I
Position of Pyrimidines and Purines in Butanol-Paper Chromatograms

Substance	Range of 80 per cent distribution in paper				Average R_F^* values, all experiments (300-350 mm.)
	Known Mixture I	Known Mixture II	Yeast nucleic acid hydrolysate	Thymus nucleic acid hydrolysate	
	mm.	mm.	mm.	mm.	
Solvent front	377	310	360	360	
Thymine	205-223	167-184		172-188	0.54
Adenine	176-195	138-159	143-163	141-161	0.45
Uracil	125-144	107-123	109-132		0.35
Cytosine	98-115	81- 96	82- 97	66- 97	0.26
Guanine	0	0	0	0	0.0
		Known Mixture IV	Known Mixture V	Known Mixture VI	
Solvent front	377	340	340	351	
Thymine desoxyriboside			160-183		0.51
Adenosine	118-139	96-113			0.33
Cytidine	56- 77		35- 61		0.16
Guanosine	35- 50	22- 41			0.10
Guanine desoxyriboside				65- 84	0.21
Xanthine					0.01
Hypoxanthine					0.18

* R_F = mm. traveled by the base per mm. traveled by the solvent past the starting line; maximum variation, ± 0.03 . Quantitative range of distribution, determined as described in a later section.

acids and from known mixtures, the absorption curves from 220 to $310\text{ m}\mu$ were determined at intervals of $5\text{ m}\mu$ in the Beckman spectrophotometer. Standard curves for authentic samples of pure bases and curves from typical samples recovered on paper strips are given in Figs. 3 to 5. The close correspondence of the data for authentic and recovered bases indicates that the substances are isolated essentially free of other ultraviolet-absorbing material. The case of guanine will be discussed more fully below.

In identification and determination of purity of bases, it has proved useful

to treat the quantitative data on the basis of ratios to a peak optical density arbitrarily taken as 1.0 at the absorption maximum. The maximum, normally around $260\text{ m}\mu$, is determined within $5\text{ m}\mu$ and all optical density values are divided by the value found at this wave-length. Expression of results in this form facilitates comparison of curves obtained with widely differing concentrations. With the spectrophotometer used the curves are reliable enough for identification of the common purines and pyrimidines

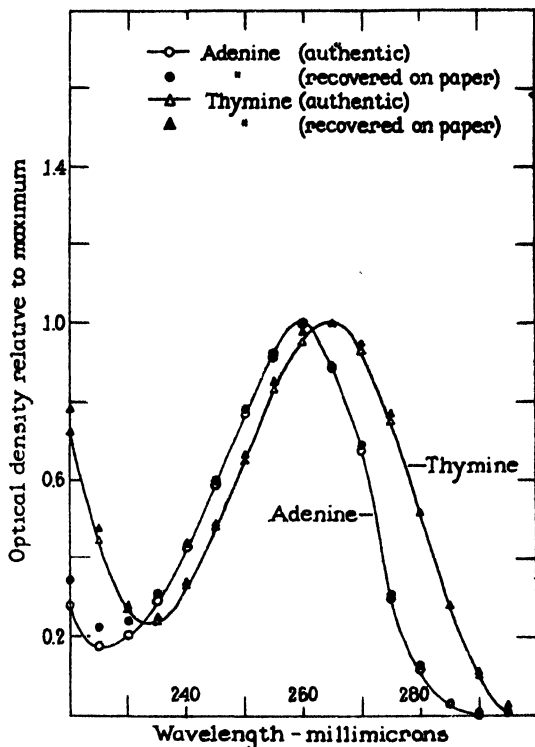


Fig. 3. Relative absorption curves of adenine and thymine in neutral solution

whenever the peak absorption is from 0.15 to 1.50 optical density units. With lower total absorption it becomes more important to make the blank corrections mentioned below.

The relative absorption data for the five bases in neutral, alkaline, and acid solutions are collected in Table II, together with similar standards for certain related compounds. These standard values are in approximate agreement with those estimated as accurately as may be done from the curves available in the literature (5-7). The absolute absorption, N_{260} , of a unit concentration is also given, and is used in the calculation of absolute

weight of a base from absorption measurements. The ratios of the maxima in alkaline and acid solution to the neutral maximum, $\text{max.}/N_{\text{max}}$, are also given and indicate the shifts in magnitude of absorption at the different acidities. These shifts are in some cases highly characteristic, and do not appear from the literature to be well known.

Not only is the correspondence between the absorption of authentic and recovered bases in neutral solution regularly as satisfactory as indicated in Figs. 3 to 5, but equally good agreement is found in data from alkaline or

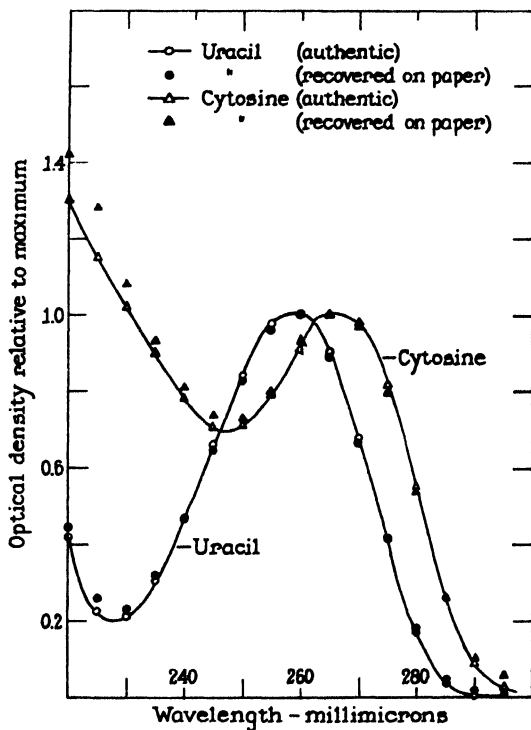


FIG. 4. Relative absorption curves of uracil and cytosine in neutral solution

acid solutions (not shown here). The recovered guanine alone is not highly pure, although it is readily identifiable. Undoubtedly the difficulty arises in part from the immobility of guanine in a flowing current of butyl alcohol, such that it will tend to remain in the same fraction with unhydrolyzed nucleotides or charred decomposition products which may be present in small amounts in hydrolysates. In any case, since guanine is extremely insoluble in water, only small amounts of it will be encountered; simple filtration or centrifugation suffices to recover a large part of the guanine present in a neutralized hydrolysate.

In precise work it is desirable to correct absorption measurements for blank errors such as cuvette absorption and the absorption of an eluate from a blank portion of the solvent-treated paper. The latter correction varies for the paper mentioned from 0.01 (optical density units) at 280 $m\mu$ to perhaps 0.03 at 220 $m\mu$ for a 10 mm. segment cut from a 40 mm. lane and may therefore be neglected in usual routine work. It is apparently due to substances having low terminal absorption in the ultraviolet range and is not materially affected by the passage of a butyl alcohol front through

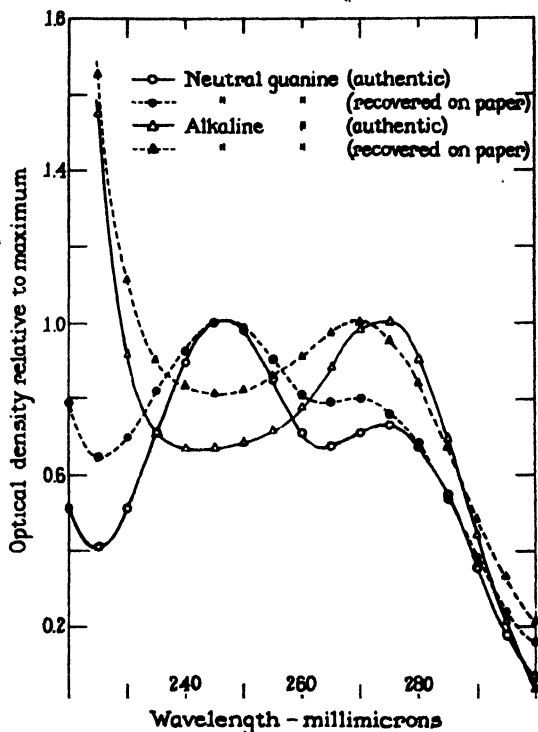


FIG. 5. Relative absorption curves of guanine in neutral and alkaline solution

the paper. Previous washing of the paper with water reduces it to some extent, but is not recommended since uneven drying appears to produce local changes in the texture and capillary properties of the paper.

The blank errors of the sort just mentioned as well as fluctuations of the pH tend to make reproducibility of the standard curves somewhat less good in the range 220 to 240 $m\mu$, even with authentic samples; therefore, undue weight is not placed upon comparisons made in this portion of the spectrum.

In Fig. 2 a minor constituent designated "epicytosine" is indicated, having a migration rate somewhat greater than that of cytosine. This small peak

TABLE II
Standard Ultraviolet Absorption Data on Purines and Pyrimidines

Wave-length, mμ	Thymine			Adenine			Uracil		
	OH	N	H	OH	N	H	OH	N	H
310	0.25	0	0	0	0	0	0.03	0	0
300	0.77	0	0	0	0	0	0.32	0	0
295	0.94	0.02	0.025	0.005	0	0	0.63	0	0
290	1.00	0.10	0.10	0.015	0	0.03	0.885	0	0.01
285	0.985	0.275	0.275	0.12	0.03	0.15	1.00	0.04	0.05
280	0.96	0.51	0.51	0.48	0.12	0.37	0.99	0.17	0.185
275	0.92	0.74	0.75	0.77	0.30	0.60	0.93	0.42	0.42
270	0.87	0.92	0.925	1.00	0.675	0.85	0.85	0.675	0.68
265	0.80	1.00	1.00	0.995	0.88	0.98	0.77	0.90	0.90
260	0.70	0.955	0.955	0.87	1.00	1.00	0.68	1.00	1.00
255	0.575	0.835	0.835	0.685	0.915	0.935	0.58	0.97	0.98
250	0.465	0.66	0.665	0.515	0.765	0.765	0.475	0.84	0.845
245	0.42	0.485	0.49	0.39	0.59	0.59	0.39	0.66	0.665
240	0.475	0.335	0.345	0.31	0.43	0.42	0.38	0.465	0.48
235	0.69	0.245	0.255	0.31	0.295	0.285	0.49	0.305	0.32
230	1.07	0.27	0.285	0.52	0.21	0.215	0.74	0.21	0.23
225	1.55	0.445	0.46	1.02	0.18	0.24	1.12	0.23	0.24
220	2.3	0.72	0.735	1.6	0.29	0.355	1.8	0.42	0.42
Max./N _{max} N ₂₀₀	0.67	(1.0) 59	0.985	0.89	(1.0) 105	0.96	0.745	(1.0) 72	1.00
	Cytosine			Guanine			Thymine desoxyriboside		
310	0.005	0	0	0	0	0.01	0	0	0
300	0.13	0.005	0.045	0.04	0.07	0.08	0.02	0.01	0.02
295	0.385	0.025	0.20	0.215	0.18	0.18			0
290	0.68	0.085	0.475	0.44	0.35	0.32	0.145	0.19	0.20
285	0.895	0.26	0.73	0.70	0.54	0.44	0.345	0.40	0.395
280	1.00	0.555	0.96	0.90	0.7	0.54	0.60	0.64	0.64
275	0.94	0.82	1.00	1.00	0.75	0.60	0.83	0.855	0.85
270	0.79	0.98	0.95	0.97	0.7	0.62	0.97	0.975	0.975
265	0.62	1.00	0.805	0.875	0.68	0.65	1.00	1.00	1.00
260	0.47	0.93	0.62	0.775	0.71	0.73	0.925	0.92	0.915
255	0.36	0.80	0.45	0.72	0.845	0.90	0.80	0.775	0.775
250	0.31	0.72	0.30	0.68	0.975	1.00	0.685	0.60	0.60
245	0.35	0.72	0.19	0.67	1.00	0.99	0.645	0.435	0.44
240	0.525	0.80	0.135	0.67	0.895	0.86	0.70	0.305	0.315
235	0.795	0.93	0.145	0.71	0.71	0.68	0.855	0.24	0.25
230	1.06	1.05	0.23	0.9	0.52	0.52	1.09	0.29	0.305
225	1.25	1.2	0.425	1.6	0.41	0.44	1.3	0.46	0.48
220	1.6	1.3	0.69		0.51	0.51	1.8	0.68	0.70
Max./N _{max} . N ₂₀₀	1.10	(1.0) 53	1.60	0.87	(1.0) 40	1.18	0.775	(1.0) 36	1.00

TABLE II—*Concluded*

Wave-length, $m\mu$	Guanine desoxyriboside			Guanine riboside			Cytosine riboside		
	OH	N	H	OH	N	H	OH	N	H
310	0	0	0.01	0	0	0.02	0	0	0.01
300	0	0.01	0.135	0.01	0.02	0.145	0.04	0.07	0.21
295	0.02	0.075	0.29						
290	0.09	0.22	0.45	0.095	0.15	0.455	0.31	0.41	0.745
285	0.305	0.42	0.59	0.305	0.43	0.585	0.59	0.675	0.935
280	0.60	0.58	0.665	0.595	0.575	0.655	0.825	0.88	1.00
275	0.845	0.665	0.69	0.835	0.665	0.685	0.975	1.00	0.96
270	0.97	0.715	0.71	0.96	0.71	0.72	1.00	0.98	0.84
265	1.00	0.75	0.795	1.00	0.75	0.82	0.93	0.89	0.67
260	0.99	0.86	0.935	0.99	0.86	0.965	0.81	0.765	0.495
255	0.96	1.00	1.00	0.965	0.995	1.00	0.725	0.655	0.34
250	0.86	0.995	0.945	0.87	1.00	0.92	0.705	0.60	0.225
245	0.71	0.895	0.785	0.70	0.895	0.74	0.74	0.60	0.155
240	0.54	0.695	0.585	0.55	0.68	0.54	0.81	0.625	0.14
235	0.42	0.475	0.40	0.44	0.475	0.36	0.88	0.675	0.185
230	0.40	0.30	0.26	0.43	0.31	0.25	0.925	0.75	0.31
225	0.61	0.205	0.24	0.66	0.22	0.27	0.99	0.815	0.50
220	1.4	0.21	0.38	1.6	0.25	0.45	1.3	0.91	0.68
$\text{Max.}/N_{\text{max.}}$ N_{260}	0.83	(1.0) 44	0.885	0.85	(1.0) 41	0.905	0.95	(1.0) 30	1.36
	Adenine riboside			Xanthine			Hypoxanthine		
310				0.06	0	0	0	0	0
300	0	0	0	0.41	0.01	0	0.01	0.01	0.01
295				0.67	0.04	0	0.04	0.03	0.03
290	0	0.01	0.05	0.89	0.12	0.04	0.08	0.06	0.06
285				1.00	0.31	0.16	0.15	0.12	0.09
280	0.16	0.165	0.23	0.98	0.59	0.39	0.31	0.18	0.14
275	0.39	0.38	0.42	0.86	0.86	0.66	0.60	0.29	0.20
270				0.70	1.00	0.91	0.84	0.43	0.32
265	0.92	0.90	0.89	0.54	0.98	1.00	1.00	0.58	0.50
260	1.00	1.00	0.995	0.43	0.86	0.97	1.00	0.75	0.70
255	0.94	0.95	1.00	0.42	0.68	0.82	0.91	0.91	0.90
250	0.775	0.805	0.84	0.48	0.53	0.64	0.75	1.00	1.00
245	0.57	0.60	0.635	0.53	0.41	0.48	0.60	0.95	0.98
240	0.40	0.42	0.44	0.57	0.36	0.41	0.46	0.79	0.86
235	0.275	0.27	0.29	0.61	0.37	0.46	0.40	0.59	0.66
230	0.24	0.18	0.23	0.78	0.39	0.51	0.48	0.40	0.46
225	0.34	0.17	0.31	1.4	0.42	0.52	0.90	0.29	0.30
220	0.8	0.37	0.58	2.8		0.52	1.8		0.23
$\text{Max.}/N_{\text{max.}}$ N_{260}	1.00	(1.0) 54	0.95	1.00	(1.0) 44 Ca.	1.02	1.00	(1.0) 47 Ca.	1.06

OH, N, and H refer to alkaline, neutral, and acid solutions, respectively; the absorption values are the ratios to a maximum taken as 1.00; $\text{max.}/N_{\text{max.}}$ relates these maxima to the neutral maximum; N_{260} at 1 mg. per cc. is the neutral absorption at 260 $m\mu$ of an absolute standard.

has been observed repeatedly in the chromatographic patterns from acid hydrolysates of a preparation of calf thymus desoxyribonucleic acid. The absorption characteristics of this material resemble those of cytosine with respect to shift in acid, alkali, etc.; nevertheless, the fraction is distinct from cytosine and is clearly not uracil. While it may be an artifact of acid-high temperature hydrolysis, its consistent appearance even at different concentrations of acid suggests that it may be a base constituent pre-existing in the nucleic acid. In this connection it might be pointed out that 5-methylcytosine was reported by Johnson and Coghill as a constituent of the desoxyribonucleic acid of the tubercle bacillus (8). Furthermore, it is true that epicytosine stands in the same relation to cytosine with respect both to its absorption spectrum and its mobility in butanol that thymine (5-methyluracil) does to uracil. More than this cannot be said until further study of epicytosine has been made.

Another abnormality that has occasionally been noted is the appearance of an "epiguanine," traveling about 0.7 as fast as cytosine and having a double band spectrum somewhat like guanine. This substance is believed to be a derivative of guanine, probably an artifact, formed in the hydrolysis of certain desoxyribonucleic acid preparations.

Routine Identification of Major Constituents—For less rigorous identification of the isolated fractions, use is made of certain characteristic features of the absorption at a few chosen points in the absorption spectra. The distinguishing features which have been used to differentiate the bases are indicated in Table III. Mention should again be made that guanine absorption responds to small fluctuations in pH; so that quantitative data for this base are not so readily reproducible.

Determination of Composition of Binary Mixtures Obtained between Absorption Peaks—In some places between the peaks, optical density of the eluate at 260 m μ may be as low as 0.10 or less, and identification of such small amounts of bases by the methods outlined heretofore would be impossible. Furthermore, if the solvent migration has not been carried far enough, or if the paper segments have been cut too wide, these regions may carry small amounts of two individual bases derived from the absorption peaks on either side of the spot in question. In case information about these weaker solutions is desired, a quantitative analysis can be made, based upon the assumption that they contain no ultraviolet-absorbing substances other than one or both of these two bases.

Simultaneous equations can be derived for any two bases at any two wave-lengths from the data in Table II. Since, however, the accuracy of such a calculation depends upon having a maximum of difference between the absorption contributions of the two components, certain wave-lengths are far more useful than others. Briefly, use is made of the following facts: thymine or uracil absorption is shifted much farther into the longer wave-

lengths than that of adenine by addition of alkali, and absorption by cytosine extends farther than that of uracil into the longer wave-lengths in neutral, and especially in acid, solution.

In the calculations that follow, total absolute absorption (corrected for paper and cuvette blanks) is indicated by the symbols used in Table III. Partial absorption calculated for a given component is further identified by

TABLE III
Characteristic Absorption Values of Individual Purines and Pyrimidines

	$\frac{N_{275}}{N_{265}}$	$\frac{N_{280}}{N_{245}}$	$\frac{OH_{290}}{OH_{260}}$	Other characteristics
Thymine	0.74	0.56	1.03	$N_{280}/N_{260} = 0.53$; $OH_{\max.}$ at 290 $m\mu$
Adenine	0.34	0.36	0.05	$N_{280}/N_{260} = 0.12$; OH shift small, H shift > in uracil or thymine
Uracil	0.46	0.32	0.89	$N_{280}/N_{260} = 0.17$; $OH_{\max.}$ at 285 $m\mu$
Cytosine	0.82	1.46	0.68	$H_{\max.} \gg N_{\max.}$; $N_{\min.} \gg H_{\min.}$ or $OH_{\min.}$; H shifts toward long wave
Guanine	1.1	0.52	0.50	2 bands, N_{245} and N_{275} ; sensitive to pH
Thymidine (desoxyriboside)	0.85	0.67	0.24	Like thymine except OH shift less; $OH_{\min.} \gg N_{\min.}$
Adenosine	0.42	0.32	0.02	Like adenine but not affected by alkali
Cytidine	1.12	1.25	0.37	Like cytosine except OH shift toward short wave; $OH_{\min.} > N_{\min.} > H_{\min.}$
Guanosine	0.88	0.35	0.16	Like guanine except OH shift smaller
Guanine desoxyriboside	0.89	0.34	0.16	Like guanosine
Xanthine	0.87	0.93	0.91	
Hypoxanthine	0.50	0.42	0.26	
2-Amino uracil (isocytosine)	1.07	0.26	0.32	

OH, N, and H refer to absolute absorption in alkaline, neutral, and acid solutions, respectively; the subscript indicates the wave-length in $m\mu$ of the maximum or minimum.

a subscript initial of the substance concerned. Thus, OH_{265T} indicates that portion of the absorption in alkaline solution at 265 $m\mu$ which is due to thymine.

(a) *Thymine-Adenine Mixtures*—For this pair the absorption is determined in alkaline solution at 290, 280, and 265 $m\mu$ (absorption at 260 $m\mu$ in neutral solution being already known).

Since $OH_{265T} = 0.79 OH_{290T}$, and since OH_{260A} is negligible, $OH_{265T} = 0.79$

OH_{290} , and $\text{OH}_{265\text{A}} = \text{OH}_{265} - \text{OH}_{265\text{T}}$ (by assumption). As a check upon these calculated values, compute $\text{OH}_{280} = 1.21 \text{ OH}_{265\text{T}} + 0.48 \text{ OH}_{265\text{A}}$, comparing this computed value with the observed total (corrected) absorption at $280 \text{ m}\mu$. Since the aim is to determine the partial absorptions at N_{260} , the following factors¹ are used: $\text{N}_{260\text{T}} = 1.78 \text{ OH}_{265\text{T}}$ and $\text{N}_{260\text{A}} = 1.12 \text{ OH}_{265\text{A}}$. The sum of these should very nearly equal the observed (corrected) N_{260} ; but even if it deviates from the latter somewhat, the ratio probably defines quite accurately the proportion of the two substances. If only one substance is present, the value for the other comes out as a very small positive or negative one. A large negative value indicates impurities not allowed for in the underlying assumption.

(b) *Adenine-Uracil Mixtures*—The procedure is the same as for the mixture just discussed, except that the numerical coefficients are different. Readings are at 290, 280, and 265 $\text{m}\mu$ in alkali. We find $\text{OH}_{265\text{U}} = 0.87 \text{ OH}_{290}$; $\text{OH}_{265\text{A}} = \text{OH}_{265} - \text{OH}_{265\text{U}}$. Checked by comparison at $280 \text{ m}\mu$, $\text{OH}_{280} = 1.29 \text{ OH}_{265\text{U}} + 0.48 \text{ OH}_{265\text{A}}$, and calculated back to neutral solution, $\text{N}_{260\text{U}} = 1.74 \text{ OH}_{265\text{U}}$ and as before, $\text{N}_{260\text{A}} = 1.12 \text{ OH}_{265\text{A}}$.

(c) *Uracil-Cytosine Mixtures*—Absorption is determined at 245, 260, and $280 \text{ m}\mu$ in neutral solution. $\text{N}_{260\text{C}} = 2.38 \text{ N}_{280} - 0.42 \text{ N}_{260}$, $\text{N}_{260\text{U}}$ being calculated by difference. To check, calculate $\text{N}_{245} = 0.775 \text{ N}_{260\text{C}} + 0.655 \text{ N}_{260\text{U}}$. More accurate results are obtained in acid solution, observations being made at 290, 265, and $245 \text{ m}\mu$: $\text{H}_{265\text{C}} = 1.73 \text{ H}_{290} - 0.019 \text{ H}_{265}$; $\text{H}_{265\text{U}} = \text{H}_{265} - \text{H}_{265\text{C}}$. Checked by $\text{H}_{245} = 0.235 \text{ H}_{265\text{C}} + 0.735 \text{ H}_{265\text{U}}$; and calculated back by $\text{N}_{260\text{C}} = 0.725 \text{ H}_{265\text{C}}$ and $\text{N}_{260\text{U}} = 1.11 \text{ H}_{265\text{U}}$.

(d) *Guanine-Nucleotide Mixtures*—Of the substances remaining in the initial spot, guanine is the principal one whose absorption is shifted by alkali. The increase in absorption at $275 \text{ m}\mu$ occasioned by adding alkali is very roughly 15 per cent of $\text{N}_{260\text{G}}$.

(e) *Other Mixtures*—Thymine and uracil are not near together on the paper and indeed appear not to be found closely associated in nature, but if necessary they may be distinguished at $310 \text{ m}\mu$ in alkaline solution.

Adenosine, adenine riboside, may occur on the paper near uracil. It may be distinguished from uracil exactly as is adenine itself, and from adenine by its total lack of absorption shift in alkali around 265 to $285 \text{ m}\mu$.

Thymidine, thymine desoxyriboside, travels in *n*-butyl alcohol very close to thymine. The nucleoside absorption is almost unaffected by alkali and may be estimated from the fact that OH_{300} of a mixture is almost entirely due to the free thymine.

Guanosine and cytidine have absorption spectra very similar to their

¹ In this and other similar calculations, the coefficients include a small empirical correction in which the 2 per cent increase of volume occasioned when 3 cc. of neutral solution were made alkaline or acid as described is automatically taken into account.

parent bases, but appear widely separated from them in the paper. Uridine has not yet been studied.

(f) *Results*—Application of the equations indicated above to mixtures encountered in an actual paper strip experiment gave the results indicated in Table IV. It will be seen that the several mixtures could be analyzed in

TABLE IV
Quantitative Analysis of Dilute Binary Mixtures

Optical density		Thymine-adenine mixtures			Uracil-adenine mixtures		
		Mixture I	Mixture II	Mixture III	Mixture I	Mixture II	Mixture III
Data	OH ₂₆₀	0.006	0.008	0.058	0.053	0.019	0.000
	OH ₂₆₆	0.195	0.072	0.051	0.067	0.063	0.059
Results	OH _{266A}	0.190	0.066	0.005	0.021	0.046	0.059
	OH _{266T}	0.005	0.006	0.046			
	OH _{266U}				0.046	0.017	0.000
Check							
Calculated	OH ₂₆₀	0.097	0.039	0.058	0.069	0.044	0.028
Observed		0.099	0.042	0.057	0.072	0.046	0.028
Calculated	N ₂₆₀	0.222	0.085	0.088	0.104	0.082	0.066
Observed		0.222	0.092	0.086	0.105	0.081	0.063
Cytosine-uracil mixtures							
		Mixture I		Mixture II		Mixture III	
Data	H ₂₆₀	0.089		0.043		0.005	
	H ₂₆₆	0.163		0.172		0.100	
Results	H _{266C}	0.151		0.071		0.007	
	H _{266U}	0.012		0.101		0.093	
Check							
Calculated	H ₂₆₆	0.045		0.091		0.070	
Observed		0.052		0.085		0.076	
Calculated	N ₂₆₀	0.122		0.163		0.108	
Observed		0.114		0.159		0.108	

The figures represent corrected optical density values, respectively. OH, N, and H refer to alkaline, neutral, and acid solutions; OH₂₆₆ represents the total absorption at 265 mμ in alkaline solution; OH_{266A}, the partial absorption due to adenine at 265 mμ in alkaline solution; etc.

terms of two partial absorption spectra which allow successful prediction of the absorption value at another reference wave-length. The broken line curves of Figs. 1 and 2 are based upon similar analyses in other cases. It may be pointed out that the solutions described in Table IV contained 0.6 to 2.5 γ (average 1.5 γ) of total bases per cc.

Quantitative Recovery of Bases—Since only negligible quantities of any

base are present outside the region immediately surrounding its peak absorption, the recovered quantity can be immediately ascertained by totaling the absorption found in this region. For such purposes it is best to analyze, as just described, all solutions which may conceivably be mixtures. The sum of total absorptions of the pure solutions and of the partial absorptions in mixtures is the absorption that would have been obtained if all the recovered base were present in one portion of eluting solvent (in this work, 3.5 cc.). This is the sum of a series of definite integrals, which could also be determined, far less accurately however, from the area under the corresponding peak on the smoothed chromatogram plot.

Table V illustrates the recovery of bases from strips carrying single compounds and also known mixtures. The figures are given in micrograms

TABLE V
Quantitative Recovery of Purines and Pyrimidines

analyzed		Thymine	Adenine	Uracil	Cytosine	Guanine
		γ	γ	γ	γ	γ
stances	Theory	90	89	94	91	
	Found	79	93	92	86	
Mixture I	Theory	84	52	66	84	
	Found	81	50	65	79	
Mixture II	Theory	72	71	75	72	
	Found	67	69	73	69	

2 mg. yeast nucleic acid hydrolyzed 2 hrs. at 120° with hydrochloric acid						
normality of acid						
0.4		0	123	7	9	125
0.7		0	112	11	9	125
2.4		0	112	54	18	140
6		0	132	117	52	125

of anhydrous bases.² There is clear evidence that the recovery is essentially quantitative, errors probably being caused in large part by some uncertainty and variability in the corrections due to substances present in the paper.

Study of Nucleic Acid Hydrolysis—As a practical test of the method, equal samples of yeast nucleic acid were hydrolyzed at 120° with varying concentrations of hydrochloric acid. The hydrolysates were concentrated to dryness *in vacuo* to remove hydrochloric acid, neutralized, and applied in known quantities to the paper. After migration with butyl alcohol, the bases were recovered to the extent shown in Table V. It will be noted that

² Since the theoretical, expected values are based upon absorption data, the agreement between theoretical and actual recoveries is independent of the specific extinction coefficients used.

free adenine is liberated rapidly at acid concentrations too low to liberate appreciable quantities of the uracil or cytosine. Appreciable quantities of nucleosides are found in such partial hydrolysates. At higher acid concentrations the nucleosides are decreased and uracil and, finally, cytosine appear in free form. The maximum amount of cytosine indicated in Table V may still represent incomplete recovery. Guanine is apparently liberated in free form as rapidly as the adenine; otherwise the nucleoside, which travels about 0.4 as fast as cytosine, would have been observed. These results are in keeping with the known greater lability of the purine ribosides compared with the pyrimidine ribosides.

DISCUSSION

The method outlined above appears to provide a means of estimating with some accuracy the constituent purine and pyrimidine bases, and probably the nucleosides, in hydrolysates of nucleic acids. The spectrophotometric standards given herewith make it possible to ascertain the identity and purity of recovered bases and the composition of binary mixtures that may be encountered. These aims have not been fulfilled to anywhere near the same extent in the paper chromatography of amino acids. Nevertheless, it should be pointed out that the present method, even when used for qualitative purposes, can be somewhat tedious, and the careful quantitative investigation of twenty or thirty strip segments for one single analysis may require several hours. Furthermore, it is not a simple matter to hydrolyze quantitatively a nucleic acid preparation without encountering some decomposition of the bases themselves. As already mentioned, two anomalous bases have been found in hydrolysates of certain desoxyribonucleic acid preparations, and one of them is believed to be a degradation product of guanine, or some other artifact. It is a fortunate circumstance that sugar constituents contribute very little to ultra-violet absorption either before or after hydrolysis.

In any case, the quantitative separation on paper strips should provide a means of judging whether the proportion of individual bases varies from one nucleic acid to another. In addition, if nucleic acids differ in the nature or arrangement of their internucleotide linkages, the rate at which individual bases are liberated during chemical or enzymatic hydrolysis may prove to be characteristic of individual modes of combination present. Investigation of residual fractions and fragments released during enzymatic hydrolysis (see (9)) should be rendered more convenient by the techniques given above.

Since this manuscript was completed, an abstract by Vischer and Chargaff (10) has appeared, indicating that these workers have now extended paper chromatography to the pyrimidines uracil and thymine.

They also now employ solvents not absorbing ultraviolet light and have applied the separation to quantitative analysis of nucleic acids. Their procedure is doubtless rather similar to the one reported here, although purines and pyrimidines are apparently determined in separate hydrolysates and in different solvents. These workers mention the conversion of cytosine to uracil during acid hydrolysis, which has so far not been observed in this laboratory, but their experience with hydrolysates of different nucleic acids appears to be more extensive at the present time.

The author wishes to acknowledge the patient and conscientious assistance of Margaret Brophy which greatly aided in the development of this method.

SUMMARY

1. By paper strip chromatography in a butyl alcohol system, cytosine, uracil, adenine, and thymine may be isolated from mixtures and from hydrolysates of nucleic acids. Guanine can also be recovered under favorable circumstances.

2. The nucleosides that have been examined, cytidine, guanosine, adenosine, and thymidine, can also be separated from each other and from most of the free bases in the same system.

3. The isolated substances can be identified and their quantity determined by ultraviolet spectrophotometry. The substances are normally recovered in highly purified form if the migration is carried far enough.

4. If binary mixtures are encountered, their composition may be quantitatively determined.

5. Chromatography of partial hydrolysates of nucleic acids reveals the rate of liberation of individual bases and nucleosides.

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MAGNESIUM PROTOPORPHYRIN AS A PRECURSOR OF CHLOROPHYLL IN CHLORELLA*

BY S. GRANICK

WITH THE TECHNICAL ASSISTANCE OF MISS RUTH KETT

(From the Laboratories of The Rockefeller Institute for Medical Research, New York)

(Received for publication, April 17, 1948)

In this paper, we wish to describe the separation and identification of another intermediate in chlorophyll synthesis from another *Chlorella* mutant.

The *Chlorella vulgaris* mutant 60 was isolated as an orange-colored colony after irradiation of normal green *Chlorella* cells with x-rays. Single cells from this colony showed constancy in their properties. The colonies which developed on the inorganic salts-glucose agar medium had a dull yellow color which turned orange-brown in 4 to 7 days when grown either in the light or dark at room temperature. At 36° growth was good but the colonies remained dull yellow. At 15° growth was very poor and colonies also remained dull yellow. The cells were grown in large flasks and harvested after 7 to 8 days. To minimize the chances of dealing with a mixed population due to spontaneous mutation, cultures for a large batch were always started from a typical colony derived from a single cell.

Extraction of Pigments from Supernatant Suspension of Cells—The cells were found to contain a complex mixture of pigments, the predominating ones being the carotenoids, with small amounts of protoporphyrin, magnesium protoporphyrin, and traces of a greenish pigment. By shaking the cells in distilled water, a reddish brown cloudy suspension was obtained which was relatively free of carotenoids and had absorption bands at 640, 590, 540 to 550, 470 to 480, and 420 to 425 m μ , as measured in a Beckman spectrophotometer (Fig. 1). Pigments from this cloudy suspension were readily isolated by treating the suspension with an equal volume of alcohol, saturating with NaCl, and extracting into ether. Preliminary tests showed that protoporphyrin was present in the ether solution. To get rid of the protoporphyrin the ether solution was washed with water several times and then rapidly extracted with an equal volume of ice-cold 1 N HCl.¹ (The acid aqueous layer was shown spectrophotometrically to contain protoporphyrin.) The ether layer was immediately treated with an equal

* This is the fourth of a series of papers on porphyrins and related compounds. For the third paper, see Granick (1).

¹ If no foaming occurs, then only a little Mg protoporphyrin will be decomposed by shaking the ether layer with cold 1 N HCl.

volume of ice-cold 1:1 solution of 0.02 N KOH and absolute alcohol. A pinkish fluorescent pigment entered the aqueous phase. (The residue in the ether now consisted of carotenoids and of a trace of a greenish pigment.)

The absorption spectrum of the aqueous alcohol solution was measured and the solution was found to contain two components (Fig. 2). The major component had two prominent bands in the visible with maxima at 550 and 588 $m\mu$ and an intense ultraviolet maximum at 418 $m\mu$; this com-

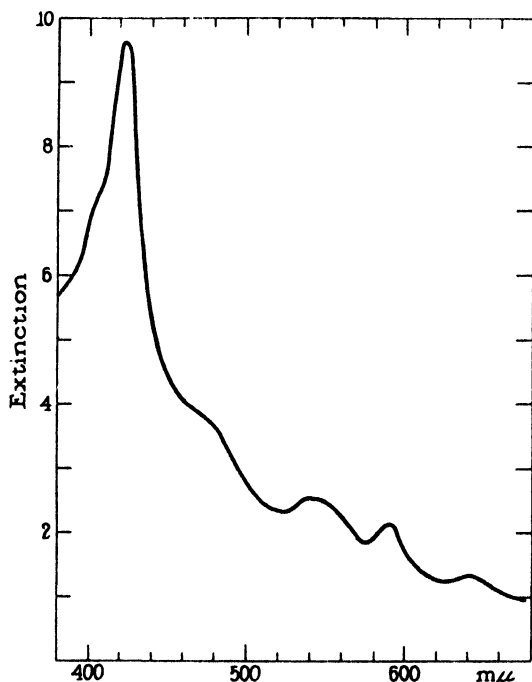


FIG. 1. Absorption spectrum of cloudy supernatant from cells, where extinction signifies observed densities.

ponent was later identified as magnesium protoporphyrin. The other, lesser component was found to be protoporphyrin which explained the bands at 630, 530, and 505 $m\mu$. The bands seen in the cloudy aqueous suspension (Fig. 1) are best interpreted as representing colloidal aggregates of protoporphyrin with a small amount of magnesium protoporphyrin (1).

Extraction of Pigments from Cells—The isolation of the pigment, having bands at 550 and 588 $m\mu$, was difficult because of the sensitivity of this pigment to acids, its low concentration, and the rather large amounts of contaminating yellow pigments. No simple procedure was found. Only

the general principles of the isolation will be described: it was necessary to control each step of the isolation by observations in the hand spectroscope.

To keep the cells slightly alkaline in order to avoid splitting out of the Mg, sodium bicarbonate was added to some 100 cc. of packed cells, and these were extracted until colorless with 80 per cent alcohol and 80 per cent acetone. To remove yellow pigments and fats and traces of green pigments, the alcoholic solutions were diluted with an equal volume of water, made alkaline with NH_4OH , and extracted with ether. The aqueous solution was brought to pH 5.5 to 6.0 with solid KH_2PO_4 and shaken with

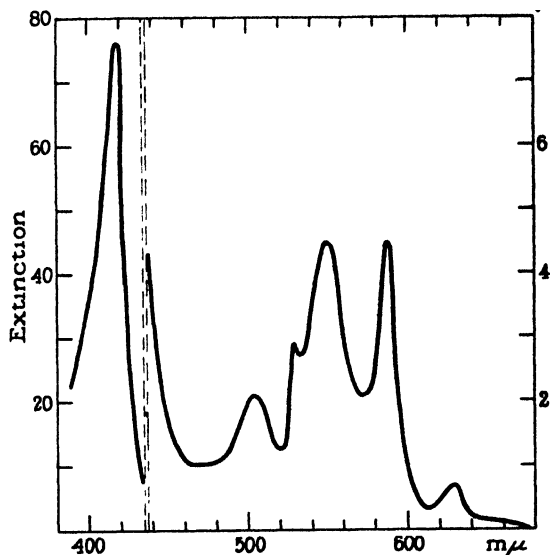


FIG. 2. Absorption spectrum of alkaline alcoholic solution derived from supernatant. The three prominent bands are due to magnesium protoporphyrin. The extinction is 10 times higher on the left side than on the right side. The extinction signifies the observed densities.

a small volume of *n*-amyl alcohol. Overnight in the ice box, the pigment was found to have collected in the amyl alcohol layer. The amyl alcohol layer was separated and evaporated to dryness under reduced pressure; the pink pigments were taken up in ether and shaken into a 50 per cent alcoholic layer containing dilute NH_4OH and again driven into ether by acidifying cautiously with acetate buffer and saturating the aqueous alcoholic layer with NaCl . This transfer between ether and aqueous alcohol was repeated twice more. The ether solution was then concentrated to 10 cc. The ether solution now contained as major components the two pigments, protoporphyrin and Mg protoporphyrin. By shaking

the ether solution with 0.05 cc. of 3 N NH_4OH , and then placing in the ice box overnight, the protoporphyrin was found to be precipitated out at the interface. (Under these conditions Mg protoporphyrin precipitated out only after 3 to 4 days.)

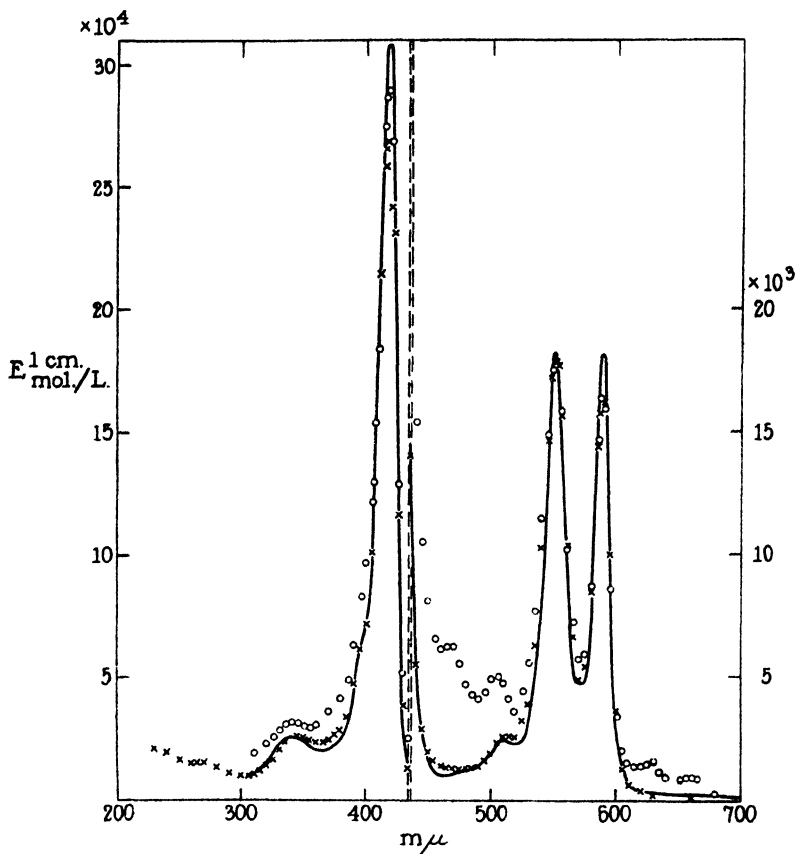


FIG. 3. Smooth curve, absorption spectrum of synthetic Mg protoporphyrin di-methyl ester in ether. $\times$, absorption spectrum of synthetic Mg protoporphyrin in 0.02 N KOH containing 50 per cent ethanol. $\circ$, absorption spectrum of pigments isolated from *Chlorella* 60, measured in 0.02 N KOH containing 50 per cent ethanol; the extinction values were adjusted at 588 $m\mu$ to the Mg protoporphyrin curve by a factor and the remaining points were multiplied by this factor.

An aliquot of this solution, evaporated to dryness, was taken up in a solution of 0.02 N KOH containing 50 per cent ethyl alcohol and measured spectrophotometrically. One point on this absorption curve (i.e. 588 $m\mu$) was adjusted to the curve for synthetic Mg protoporphyrin by a factor, and the remaining points were multiplied by this factor (Fig. 3). From

Fig. 3 it is seen that the predominating pigment in this solution corresponds to synthetic Mg protoporphyrin with respect to the positions of the absorption maxima (418, 551, and 589 $m\mu$) and the relative heights of the bands. The solution is still contaminated by small amounts of protoporphyrin, as seen in the bands at 505 and 630 $m\mu$, possibly by carotenoids (*i.e.* band at 465 $m\mu$), and by a trace of a greenish pigment (640 to 670 $m\mu$). Since the solution was estimated to contain only about 0.5 mg. of Mg protoporphyrin, further purification by chromatographing was not attempted.

Identification of Porphyrin of Metal Complex As Protoporphyrin—Since only about 0.5 mg. of the magnesium protoporphyrin was isolated from *Chlorella*, and since the isolation in the crystalline state would have been too tedious, it was deemed necessary to obtain supporting evidence for the composition of this compound by identification of the kind of porphyrin and the kind of metal. An aliquot of the ether solution was extracted with 3 N HCl. At this acidity the metal was split off and all of the pink pigment entered the aqueous phase. The aqueous solution was neutralized and the porphyrin reextracted into ether. The ether solution was washed with water and then extracted successively with increasing concentrations of HCl. No porphyrins were extractable from ether with HCl solutions below 0.1 N. Two fractions were isolated by extraction between 0.1 and 0.4 N HCl and between 0.4 and 1.0 N HCl. The absorption spectra of both these fractions fell, within experimental error, on the curve of pure protoporphyrin (Fig. 4). This result indicates that neither a monovinyl nor any other porphyrin except protoporphyrin was present, and therefore the pigment originally isolated must be a derivative of protoporphyrin. Neither esters of magnesium protoporphyrin nor esters of protoporphyrin could be found in this preparation or in crude preparations that had been extracted from cells in which the use of alkaline fluids was avoided. Such esters would have been detected in the ether after extraction with 1 N HCl. (When *Chlorella* 60 was grown in a medium containing 1 mg. of Cu per liter, a small amount of pigment was observed which was stable in strong HCl; the positions of the band maxima were those of Cu protoporphyrin.)

Identification of Magnesium As Metal in Complex—The qualitative identification of magnesium was carried out with the quinalizarin reagent (2). The test is highly specific, only beryllium and lanthanum besides magnesium being reported to form a blue precipitate in strongly alkaline solution. However, the test is not particularly sensitive. A method was devised to remove the porphyrin, which interferes with observation of the blue precipitate, and at the same time to keep the magnesium as concentrated as possible.

The test was carried out in the following way: 5 cc. of the ether solution, estimated to contain approximately 250 γ of magnesium protopor-

phyrin, were placed in a 15 cc. conical centrifuge tube. Then 0.10 cc. of 4.6 N HCl was added, and air was bubbled through to stir the HCl into the ether layer. After several minutes the ether layer became completely colorless and all of the protoporphyrin was now collected into the drop of aqueous HCl at the bottom of the tube. The HCl was neutralized by adding 0.12 cc. of 4.00 N NaOH plus 0.01 cc. of glacial acetic acid to the tube. Air was again bubbled through until all of the protoporphyrin had passed back into the ether layer. The tube was then centrifuged. The clear colorless aqueous droplet at the bottom of the tube was now removed

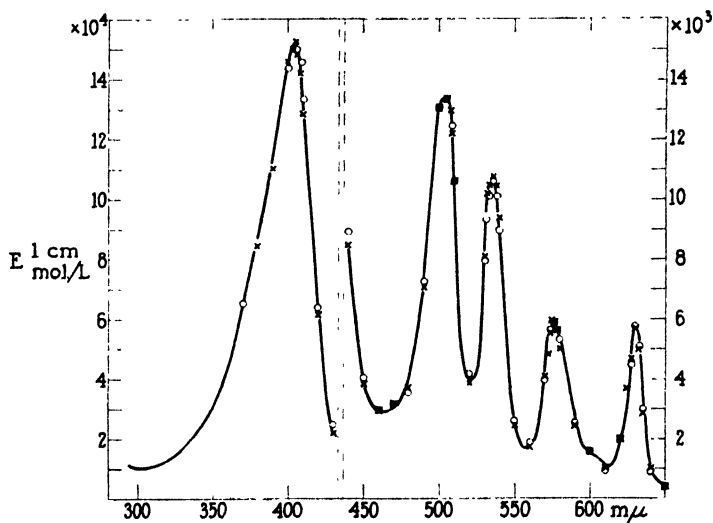


FIG. 4. Smooth curve, absorption spectrum of synthetic protoporphyrin IX in ether. Absorption of porphyrin derived from magnesium protoporphyrin isolated from mutant *Chlorella*; X, porphyrin extracted between 0.1 and 0.4 N HCl; O, porphyrin extracted between 0.4 and 1.0 N HCl. The extinction is 10 times higher on the left side than on the right side.

with a capillary pipette and placed on a drop plate, and 2 drops of alcoholic quinalizarin (10 mg. per cent) and 2 drops of 2 N NaOH were added. Blue granules appeared within 1 minute. At the same time and on the same drop plate a series of known concentrations of Mg^{++} was run, including controls of the reagents. From the rate at which the blue granules appeared and their volume it was estimated that the quantity of Mg^{++} was about 10 γ , which was in the predicted range if the compound was Mg^{++} protoporphyrin. (At this concentration Ca^{++} does not give a characteristic blue precipitate.)

Neither magnesium protoporphyrin nor its ester has ever been prepared

in the crystalline form. It was deemed necessary to prepare the magnesium protoporphyrin and to study its properties, in order to compare it with the pigment derived from the *Chorella* mutant. The dipotassium salt of magnesium protoporphyrin was made by way of the dimethyl ester.

Preparation of Magnesium Protoporphyrin Dimethyl Ester—The method used here, of inserting the magnesium into protoporphyrin by means of a decomposed Grignard reagent, is a modification of that used by Fischer and Dürr (3). To obtain this compound in its crystalline form, it was found necessary to use highly purified protoporphyrin ester, to avoid temperatures above 80°, and to run the reaction preferably in the absence of O₂.

In a 500 cc. round bottom triple necked flask was placed 1.0 gm. of Mg ribbon. This was washed by decantation with anhydrous ether. Then 30 cc. of anhydrous ether and 15 cc. of ethyl bromide were added. The flask was connected to a reflux condenser with a drying tube attached, and the contents warmed gently. After some 20 minutes the reaction was ended. To distil off the ether and excess ethyl bromide most easily, the water in the reflux condenser was emptied, and the flask, still attached to the condenser, was placed in hot water. Toward the end of the evaporation, suction was applied through the drying tube to aid in the distillation and drying.

The flask containing the dry ethyl magnesium bromide was cooled in ice water, and 75 cc. of dry *n*-propyl alcohol (distilled over CaO) were added in small portions through the top of the reflux condenser. The residue dissolved completely. The solution was then heated and refluxed for 10 minutes to decompose the last traces of the Grignard reagent. After cooling to 50°, 280 mg. of twice crystallized protoporphyrin dimethyl ester were added to the flask, arrangement being made to pass dry N₂ slowly into one arm of the flask. The flask was placed on a water bath and kept at 70–75° for 5 to 7 hours or until the protoporphyrin band at 630 m μ had disappeared. Higher temperatures or prolonged heating led to yellow decomposition products.

The material in the flask was now transferred, with the aid of a small amount of ether, to a Claisen distilling flask and the propyl alcohol distilled almost to dryness under diminished pressure in the presence of N₂. The dry material was now transferred with the aid of 750 cc. of water and 750 cc. of ether to a 2 liter separatory funnel. The solution was shaken to extract most of the Mg protoporphyrin ester into the ether. Then 100 cc. of a solution containing 10 gm. of ammonium acetate and 10 gm. of Na₂HPO₄ were added. A flocculent precipitate of MgNH₄PO₄ was produced. This was drawn off and the ether layer washed several times with water. A small amount of impurity went to the interphase and was drawn off. The ether solution was dried with anhydrous sodium sulfate and evaporated

to dryness under diminished pressure in the presence of N_2 . The dark red powdery residue was dissolved in some 30 cc. of wet ether and filtered. A small brownish residue with a band at 470 to 480 $m\mu$ collected on the filter and was discarded.

Even when pure, the Mg protoporphyrin ester is difficult to crystallize from solution, although crystals will be found to form on a glass slide under the microscope. Crystallization was accomplished in the following manner: To the concentrated ether solution, 3 cc. of xylene were added, and the solution further evaporated down to about 7 to 10 cc. Then 5 cc. of low boiling petroleum ether were added (b.p. 30–60°) and a crystalline precipitate rapidly formed. After cooling for several hours the precipitate was centrifuged, washed with low boiling petroleum ether by centrifuging, and then filtered off. The yield of this crystalline pinkish powder was 220 mg. or about 75 per cent of theory.

A portion of the pink powder was washed on the filter with anhydrous ether. The filtrate consisted of a colloidal solution; a slight residue remained on the filter paper. To the filtrate was added low boiling petroleum ether, and a precipitate of plates and highly twinned crystals resulted. This was centrifuged, washed with low boiling petroleum ether, and dried *in vacuo*. Analyses of this material showed the following percentage composition.

$C_{36}H_{36}O_4N_4Mg$.	Calculated.	C 70.5, H 5.89, N 9.15, Mg 3.98
	Found.	" 70.36, " 6.01, " 9.04, " 4.00

An aliquot of this crystalline material was dissolved in moist ether and the absorption spectrum of the red fluorescent pigment was determined (smooth curve, Fig. 3). The absorption bands are very sharp and high. The molar extinction per cm. of light path for a given wave-length is given by

$$E_{\text{mole per liter}}^1 \text{ cm.} = \log_{10} \frac{I_0}{I} \cdot \frac{1}{\text{cm.} \times \text{mole per liter}}$$

E at 419 $m\mu$ = 308,000, at 340 $m\mu$ = 20,550, at 551 $m\mu$ = 18,200, at 589 $m\mu$ = 18,200, and at 510 $m\mu$ = 2450.

A comparison of the position of the visible absorption spectra of various divalent metal mesoporphyrins which have been studied shows that the Mg compound has its bands furthest displaced toward the red end of the spectrum (4).

Preparation of Dipotassium Salt of Mg Protoporphyrin—30 mg. of the Mg protoporphyrin ester were treated in a 50 cc. centrifuge tube with 5 cc. of 30 per cent methyl alcoholic KOH for 15 minutes at 40°. Then 10 to 15 cc. of water were added, resulting in a flocculent precipitate. The precipitate was centrifuged down and the supernatant liquid discarded. The

precipitate was dissolved in 5 cc. of hot methanol and placed in the ice box. Crystals arose, consisting of rhomboid plates, often highly twinned, especially if rapidly formed (Fig. 5). If crystallization did not occur under these conditions, then crystallization could be induced by adding small portions of a solution made up by diluting the methyl alcoholic KOH 1:10 with water. The plates were dichroic, dark red and pale yellow. On the basis of $K_2C_{34}H_{30}O_4Mg$, calculated, N = 8.4 per cent; found, 8.2 per cent. The absorption spectrum of this compound was measured in 0.02 N KOH containing 50 per cent ethanol. The extinction values in this solution are lower than for the ester in the ether solution (Fig. 3).

Biological Activity—*Hemophilus influenzae* Turner requires heme or protoporphyrin for growth and for the reduction of nitrate to nitrite (4).

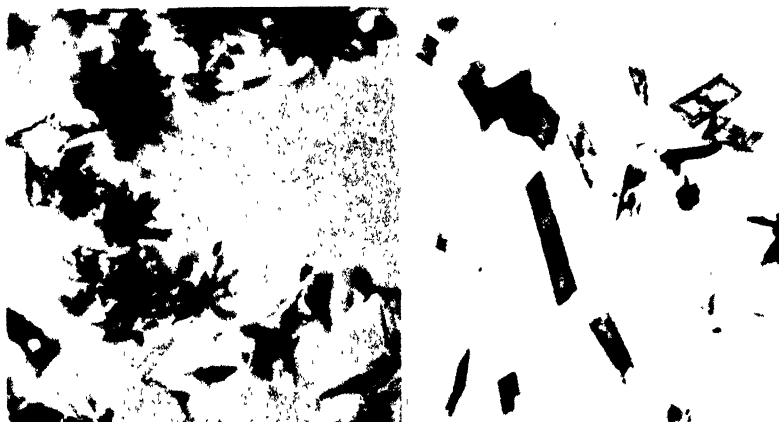


FIG. 5. Crystals of the dipotassium salt of magnesium protoporphyrin. Left-hand, formed rapidly from aqueous methanol, $\times 400$; right-hand, formed slowly from methanol, $\times 100$.

It was found that Mg protoporphyrin would support the growth of this organism at a concentration one-fifth of that of protoporphyrin. As with protoporphyrin, the nitrate reducing activity of organisms grown on Mg protoporphyrin was proportional to the growth of the organisms. A tentative explanation for the fact that the growth-promoting effect of magnesium protoporphyrin is greater than that of the protoporphyrin itself may be found in the fact that the magnesium porphyrin has a smaller tendency to form colloidal solutions and will therefore be more readily available to the cell than porphyrin itself. Once in the cell, it is probable that the magnesium is split out and then the iron inserted (4).

We desire to express our thanks to Dr. L. Michaelis for his constant stimulation and advice.

SUMMARY

From 100 cc. of cells of *Chorella* mutant 60, about 0.5 mg. of a pinkish fluorescent pigment was isolated. This was identified as Mg protoporphyrin in several ways. Its absorption spectrum agreed with the spectrum of synthetic magnesium protoporphyrin in position and relative heights of the bands. The metal was split out of the complex with acid and identified as Mg^{++} by the quinalizarin lake method. The porphyrin was identified as protoporphyrin by its HCl number and by its absorption spectrum.

Methods for the synthesis of magnesium protoporphyrin dimethyl ester and of the dipotassium salt of magnesium protoporphyrin are described.

The isolation of magnesium protoporphyrin suggests that, after the synthesis of protoporphyrin, insertion of magnesium is the next step in the biological synthesis of chlorophyll by *Chlorella*.

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METHODS FOR DETERMINING THE IRON CONTENT OF MILK

By FRANCES A. JOHNSTON, NAOMI GELLMAN, AND JUNIATA STROM

(From the College of Home Economics and the School of Nutrition, Cornell University, Ithaca)

(Received for publication, April 23, 1948)

The iron content of milk was reported as 2.0 mg. per kilo in the mimeographed "Tables of food composition" published by the National Research Council (1) in 1943. In 1945, when the "Tables" were republished in bulletin form by the Bureau of Human Nutrition and Home Economics in conjunction with the National Research Council (2), the value was dropped to 0.7 mg. per kilo. This value is still about twice as large as that of 0.32 mg. per kilo reported by the senior author (3) in 1944. Thus the present study was undertaken to check the validity of the method used in the 1944 investigation. In view of the fact that milk is used in large quantities, a correct value for its iron content is important. Incidental to this study, a method has been developed which is deemed more satisfactory than the one used in the 1944 study.

EXPERIMENTAL

In the 1944 study the milk was dry-ashed and iron determined by the thiocyanate method. 50 ml. of milk were mixed with 15 drops of nitric acid in a platinum dish, the curdled milk was heated at 80° until the water had evaporated, the residue was then placed in a muffle furnace in which the heat was slowly increased to 400°, and heating continued until the ash was white. The ash was dissolved in redistilled hydrochloric acid and filtered through No. 44 Whatman filter paper. The filter paper containing the incompletely ashed material was dried, fused with sodium carbonate, and dissolved in redistilled hydrochloric acid. The combined ash solutions were put into an Erlenmeyer flask, 15 drops of concentrated nitric acid were added, and the flask was heated on a hot-plate to hydrolyze pyrophosphates to orthophosphates. Heating was continued for 30 minutes or enough longer to reduce the volume to approximately 4 ml. The solution was diluted to 50 ml. and a 20 ml. aliquot used for the iron determination. Stugart's (4) thiocyanate method was employed and the colors were read in an Evelyn colorimeter.

The method was checked in the following ways.

(a) Samples of milk larger and smaller than the 50 ml. originally used were analyzed. The iron content of 25, 50, and 75 ml. samples was found to be 0.46, 0.42, and 0.42 mg. per kilo respectively. The agreement is satisfactory for small amounts of iron.

(b) To check for the possibility of volatilization of iron during dry ashing at 400°, recoveries could not be used because the salt added for the recoveries would not be in the same form as that in the milk. Two ways of testing were considered feasible. In one the milk was wet-ashed by a modification of the method of Roberts, Beardsley, and Taylor (5), in which case the temperature would be no higher than 340°; the results were compared with the same milk dry-ashed by the original method at 400°. Accordingly, milk from three companies was analyzed by both procedures. By the wet ashing procedure the iron content was found to be respectively 0.29, 0.31, and 0.27 mg. per kilo and by the dry ashing procedure 0.28, 0.28, and 0.28 mg. per kilo. This showed that the high temperature used in dry ashing

TABLE I
Iron Content of Milk As Found by Three Methods

Distributor	Bottle No.	Dry-ashed thiocyanate	Dry-ashed α, α' -dipyridyl	Pptd. α, α' -dipyridyl
		<i>mg. per kg.</i>	<i>mg. per kg.</i>	<i>mg. per kg.</i>
A	1	0.32	0.33	0.31
"	2	0.34	0.34	0.35
B	1	0.32	0.30	0.32
"	2	0.30	0.33	0.25
C	1	0.36	0.35	0.27
"	2	0.46	0.47	0.34
"	3	0.57	0.59	0.50
D	1	0.37	0.42	0.32
"	2	0.41	0.42	0.35
E	1	0.39	0.40	0.33
Average . .		0.38	0.39	0.33

does not cause a loss of iron, or at least causes no more loss than occurs at the lower temperature used in wet ashing.

The second means of testing for the possibility of volatilization of iron during dry ashing was to employ a new method developed by Ruegamer, Michaud, and Elvehjem (6) in which milk is not ashed at all and, therefore, not subjected to a high temperature. The protein is precipitated with trichloroacetic acid and the α, α' -dipyridyl test applied to the filtrate. When the iron content of ten bottles of milk was determined by our dry ashing method and by the non-ashing method, the values for three of the bottles (see Table I) agreed within ± 0.01 mg., but the values for seven of the bottles found by the non-ashing method were actually 0.05 to 0.12 mg. per kilo lower than those found by our method. This does not indicate any loss of iron during ashing by our method. When the thiocyanate

color test and the α, α' -dipyridyl color tests were applied to the ashes obtained by our method of dry ashing, the difference in the mean values was only 0.01 mg. per kilo (Table I). The difference in the values found by our dry ashing method and the non-ashing method was, therefore, attributed to the processes used for the removal of organic material rather than to the color tests.

(c) The possibility that iron might be lost during hydrolysis was considered also. Since the milk ash was dissolved in hydrochloric acid, some of the iron present in the resulting solution may have been in the form of ferric chloride, although much of the iron was present as phosphate. It is this ferric chloride that might be carried off during the hydrolysis of the pyrophosphate to orthophosphate. To test this possibility a known amount of ferric chloride was added before hydrolysis. Recoveries of 91.1 and 104.3 per cent indicated little or no loss during hydrolysis.

Improved Method of Ashing

In the course of checking our 1944 method by a comparison of values found by wet ashing and dry ashing, a method for wet ashing was developed which was deemed more satisfactory than the original dry ashing or any other dry ashing method because it obviated much of the danger of contamination. This is an important point, as it is possible to get fairly even contamination, so that duplicates check even when first starting dry ashing procedures. Thus values higher than the true ones may be found from the first two or three analyses; this is not apt to happen with wet ashing. Wet ashing methods have been considered unsuitable for use with milk by some investigators. We believe, however, that the method we have used is suitable. We avoided the use of perchloric acid because in our experience wet ashing procedures involving its use yield values which are too low.

The method we used is a modification of the method of Roberts, Beardsley, and Taylor (5). It is as follows: Deliver 15 ml. of milk from a pipette, calibrated to deliver a known weight of milk, into a 300 ml. Kjeldahl flask containing Pyrex glass beads. Add 1 ml. of concentrated sulfuric acid and 5 ml. of concentrated nitric acid (0.0000 per cent iron). Heat the milk with the acids until it chars. Make three more additions of 5 ml. of nitric acid and then 0.5 ml. additions until the contents of the flask are clear and completely colorless. Cool slightly, add 1.0 ml. of superoxol dropwise, and heat until the heavy fumes disappear. It is important that all the superoxol is dispelled. Cool slightly, add 5 to 6 ml. of glass-distilled water, and let stand overnight.

Add about 5 ml. more water and filter through Whatman No. 44 filter paper in Pyrex funnels into 60 ml. pear-shaped Pyrex separatory funnels. Wash four times, keeping within a total volume of approximately 20 ml.

Add 1 drop of 0.04 potassium permanganate and mix; add more if the color fades. Add 15 ml. of isoamyl alcohol and 5 ml. of 20 per cent potassium thiocyanate. Shake, pour off the alcohol layer, and read its depth of color in an Evelyn photoelectric colorimeter against pure isoamyl alcohol set at 100 per cent transmission with Filter 490.

The ash solution is very acid, due to the 1 ml. of sulfuric acid present, but the milk ash reduces the acidity somewhat and may have some buffering effect. At any rate, the acidity does not cause turbidity in the alcohol. If the separatory funnel is gently swirled so that the contents wash the top after the water layer has separated, and if a little of the alcohol layer is discarded and the rest poured over the same place in the neck of the separatory funnel into the colorimeter tube, turbidity seldom develops. If it does, the alcohol layer can be poured through a tiny bit of hydrochloric acid-washed cotton in a small Pyrex funnel. The dry cotton removes the water which causes the turbidity.

The standard curve is constructed from readings obtained on a series of solutions, each of which contains an ashing blank and a known amount of ferric salt. For the ashing blank, 1 ml. of sulfuric acid, 21 ml. of nitric acid, and 1 ml. of superoxol are heated as for the unknowns. The resulting solution is so acid that it causes turbidity in the color test. The acidity, therefore, must be reduced with concentrated ammonium hydroxide. A high grade of ammonium hydroxide contains so little iron as to be negligible in the amount used. The amount of ammonium hydroxide to be added must be determined each day. Add the amount which is needed to neutralize 0.5 ml. of the sulfuric acid when titrated with phenolphthalein as the indicator. Add that amount to the contents of the Kjeldahl flask; cool, transfer to a separatory funnel, add the standard with care to keep the total volume at approximately 20 ml., and proceed with the color test as for the milk ash. If nine ashing blanks are prepared, triplicates can be determined for 3.0, 5.0, and 7.0 γ of iron.

The precipitate caught on the filter paper can be tested to show that it contains no iron by dissolving it in redistilled 7 N hydrochloric acid, diluting to an acidity of 1 to 2 N, and color-testing it with only about 2 ml. of isoamyl alcohol.

DISCUSSION

None of the ways of checking the method used in our 1944 study (3) for analyzing milk for iron gave any indication that the values found by that method were too low. The possibility that all the pyrophosphate is not hydrolyzed to orthophosphate by boiling with hydrochloric and nitric acids was investigated by Stugart (4), who added pyrophosphate before hydrolysis and obtained complete recoveries. Other investigators have found

values similar to our value of 0.32 mg. per kilo: Krauss and Washburn (7) found 0.34 to 0.43 mg. per liter, Roberts *et al.* (5) 0.42 mg. per kilo, and Dahlberg and Carpenter (8) 0.29 to 0.46 mg. per kilo.

SUMMARY

1. All employed methods of checking failed to indicate that the previously reported (3) value for iron in milk of 0.32 mg. per kilo was too low, despite the fact that the value of 0.7 mg. per kilo subsequently was used by others (1) in important tables.

2. A modification of the method of Roberts, Beardsley, and Taylor (5) has been described. It employs wet ashing, which lessens the danger of contamination and appears to be the most satisfactory method for the determination of iron in milk.

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THE TRANSFER OF IMMUNITY TO THE NEW-BORN CALF FROM COLOSTRUM

By EMIL L. SMITH* AND AUGUST HOLM

(From the Biological Laboratories, E. R. Squibb and Sons, New Brunswick)

(Received for publication, April 26, 1948)

Some years ago, Theobald Smith and his associates (1) showed that passive immunity acquired by the new-born calf from colostrum is of prime importance in protection against infectious disease. At about the same time, Orcutt and Howe (2) observed that, after the ingestion of colostrum, agglutinins appear in the calf serum associated with a globulin which is precipitable at low concentrations of sodium sulfate. Jameson, Alvarez-Tostado, and Sortor (3) later found by electrophoretic analysis that the serum of the new-born calf does not contain γ -globulin, and that the appearance of slow moving globulin follows the feeding of colostrum. Similar observations have been made by San Clemente and Huddleson (4) for the calf, and by Polson (5) for the new-born foal.

Since it has already been demonstrated that the immune lactoglobulin of the colostrum differs from the γ -globulin of cow serum in electrophoretic mobility and other properties (6-11), it was of interest to ascertain whether the new protein which appears in calf serum after it receives colostrum possesses the same electrophoretic properties as the colostrum globulin, or whether this immune lactoglobulin is modified during its passage from the intestine of the calf to the blood stream. Hyperimmunization was performed with a mixture of antigens: diphtheria toxin (or toxoid), vaccinia virus, and a killed culture of *Hemophilus pertussis*.¹ It was, therefore, possible to follow changes in the acquired immunity of the calf parallel with electrophoretic studies of the serum, and to determine the duration of the passively acquired immunity.

A few observations have also been made on the electrophoretic patterns of the serum of the new-born lamb before and after the ingestion of colostrum, and these have been compared with the pattern obtained with maternal sheep serum.

* Present address, Department of Biological Chemistry, University of Utah School of Medicine, Salt Lake City 1, Utah. The work reported in this paper was completed in April, 1946.

¹ Studies on the hyperimmunization of bovines with these and other antigens will be reported elsewhere by one of us (A. H.). It should also be noted that previous studies (6-11) of bovine immune proteins were made with animals immunised to these same three antigens.

Electrophoretic Studies on Calf Serum

By fractionation of hyperimmune bovine plasma, two different proteins associated with immune activity have been isolated: a γ -globulin with an electrophoretic mobility of 1.3×10^{-5} sq. cm. per volt per second, and a T component with a mobility of 2.5×10^{-6} as measured in 0.1 M veronal (diethyl barbiturate) buffer at pH 8.6 (6). Hess and Deutsch (12) have obtained similar proteins from normal bovine serum. The immune lactoglobulin of colostrum has an electrophoretic mobility similar to that of the T component (6). Since both the colostrum and T-globulins have mobilities near that of fibrinogen, our observations were made with serum rather than with plasma. The electrophoretic analyses were performed at 1° in a Tiselius apparatus equipped with the Longworth schlieren scanning device. The calf was separated from the mother immediately after birth and was fed 2750 cc. of the first colostrum during the next 40 hours; thereafter, the calf was fed on a mixed artificial diet.

Fig. 1 shows the electrophoretic patterns of the sera of the calf at birth and at later intervals. For comparison, the serum of the mother taken 3 days before term is also shown. It is apparent from Fig. 1 that the serum of the new-born calf does not contain γ -globulin and that T-globulin is also absent. Moreover, none of the antibodies present in the serum of the mother could be demonstrated in the serum in the new-born (Table III). This is in agreement with previous observations that there is no placental transfer of antibodies in this species (13, 14). After ingestion of colostrum, a new component appears in calf serum which has an electrophoretic mobility of 2.1×10^{-5} sq. cm. per volt per second in veronal buffer of pH 8.4. Thus, this component retains the characteristic mobility of the immune lactoglobulin after passage from the digestive tract to the blood stream of the calf and is not a γ -globulin. Examination of the data of Jameson, Alvarez-Tostado, and Sortor (3) and of San Clemente and Huddleson (4) shows that in these cases also the new globulin in calf serum possesses the mobility of the colostrum globulin.

Tables I and II present the analytical data for the calf sera at different ages. These data indicate that several striking changes occur during the growth of the young calf. The most obvious is the large amount of immune protein which appears after the feeding of colostrum and its gradual diminution. Also noteworthy is the absence of γ -globulin at birth and its gradual appearance only many weeks later. The α_2 component, which decreases in concentration, is probably the fetuin described by Pedersen (15) as the main constituent of fetal α -globulin. In Table II are the values for the absolute concentration of each serum component. These data show clearly that after ingestion of colostrum there is no immediate change in the absolute amounts of the albumin or the α -globulins, but there seems to be an increase in the β_2 component. The α_2 -globulin appears late, and,

although it is first clearly separable at 122 days, it is apparently included with the α_2 at 87 days.

Both the α_2 -globulin and the albumin show a striking decrease in electrophoretic mobility after the ingestion of colostrum. This change may be due to the influence of the large amount of colostrum globulin on the viscosity and the electric properties of the diluted serum, rather than to a sudden change in the character of the α_2 -globulin or the albumin.

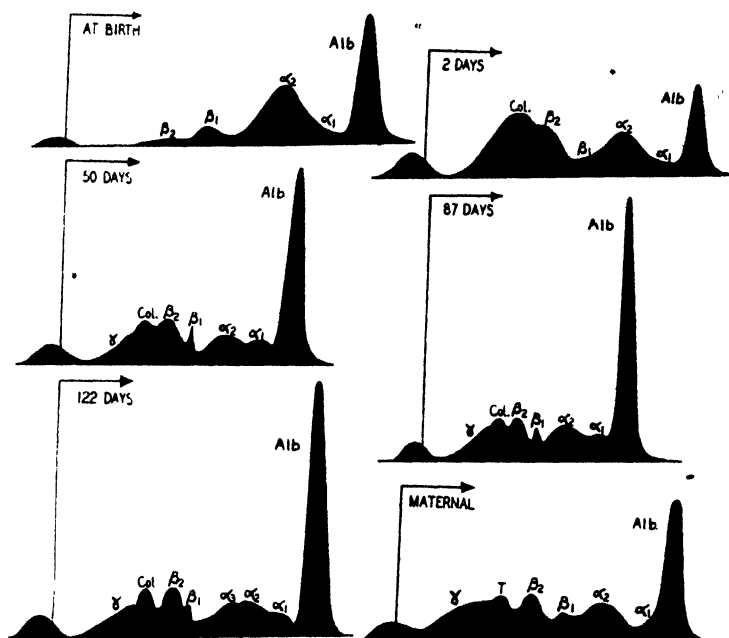


FIG. 1. Electrophoretic patterns of the descending boundaries of the serum of a new-born calf and of the same animal 2, 50, 87, and 122 days later. For comparison, the maternal serum obtained 3 days before term is also shown. The runs were performed at 1° in a veronal buffer of pH 8.4 to 8.6 and at an ionic strength of 0.1. The serum of the new-born is practically devoid of slow moving globulins. The immune component appears after feeding colostrum. The absolute heights of the different serum samples cannot be compared, as electrophoresis was performed at different protein concentrations.

In addition to the data already presented, observations were made on the sera of two other new-born calves. The electrophoretic patterns obtained on the sera before and after feeding were similar to those described above.

Immune Properties of Calf Serum

In Table III are given the data for the immune activity of the whole colostrum whey and of the calf serum at different ages. Assays of diph-

theria antitoxin were performed by the rabbit skin test as described by Jensen (16). The values for pertussis antibodies were measured in mouse-

TABLE I
Electrophoretic Analyses of Calf Serum

The electrophoretic analyses were performed at 1° in veronal buffer of pH 8.4 to 8.6 and at an ionic strength of 0.1. The mobilities are $\times 10^{-5}$ sq. cm. per volt per second and are negative in sign. The values were obtained from photographs of the descending patterns taken at 150 to 250 minutes. The serum protein was calculated from protein N $\times 6.25$.

Age of calf	Protein in serum	γ		Colostrum globulin		β_2		β_1		α_3		α_2		α_1		Albumin	
		u	Con- cen- tra- tion	u	Con- cen- tra- tion	u	Con- cen- tra- tion	u	Con- cen- tra- tion	u	Con- cen- tra- tion	u	Con- cen- tra- tion	u	Con- cen- tra- tion	u	Con- cen- tra- tion
		per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	per cent	
days	per cent		per cent		per cent		per cent		per cent		per cent		per cent		per cent		per cent
0	4.5					2.5	2	3.7	8			5.3	44	6.4	3	7.5	43
2	8.0			2.1	44	3.1	8	3.6	2			4.5	23	5.6	2	6.4	21
50	6.1	1.2	1	2.1	14	2.8	15	3.6	4			4.5	15	5.4	5	6.3	46
87	6.2	1.4	6	2.3	12	2.8	10	3.4	4			4.3	17	5.2	4	6.2	47
122	5.8	1.3	7	2.1	10	2.7	11	3.2	2	4.0	10	4.6	10	5.2	4	6.0	46
Mat- ernal		1.4	20	2.4*	10	3.0	12	3.6	5			4.7	18	5.6	3	6.3	32

* T-Globulin.

TABLE II
Composition of Calf Serum at Different Ages

The values are calculated from the percentage for each component of the total refractive increment and the total protein content of the serum. The concentrations are in gm. per 100 cc. of serum.

Age	γ	Colostrum globulin	β_2	β_1	α_3	α_2	α_1	Albumin
days								
0		0.0	0.09	0.36		1.98	0.14	1.94
2		3.52	0.64	0.16		1.84	0.16	1.68
50	0.06	0.85	0.92	0.24		0.92	0.31	2.81
87	0.37	0.74	0.62	0.25		1.05	0.25	2.91
122	0.41	0.58	0.64	0.12	0.58	0.58	0.23	2.67

protective units by a modification of Silverthorne's method (17), and the vaccinia assays were made by a rabbit skin test.²

These data show that the calf may acquire a high level of immunity from

² Holm, A., unpublished.

the colostrum, as already demonstrated with other antibodies. Since the calf was fed colostrum for only a short time, it is possible to calculate the approximate duration of the passively acquired immunity. For the pertussis and vaccinia antibodies, the time for the activities to decrease to one-half their initial values is about 50 days. The more precise determinations of the diphtheria antitoxin show a half life of about 16 days, and the data in Table II from the Tiselius analyses indicate 20 days. Since the animal was growing rapidly, dilution of the antibodies must have occurred, and the true half times must be even greater than the calculated values.

While there is some variation for the different antibodies, there is no doubt that the passive immunity is effective for a considerable length of time. It may be remarked that humans who are negative to the Schick test are regarded as immune to diphtheria when this test reflects a serum

TABLE III
Immune Properties of Colostrum and of Calf Sera at Different Ages

Age	Diphtheria antitoxin	<i>Haemophilus pertussis</i> antibody	Vaccinia virus antibody*
days	units per cc.	units per cc.	
0	<0.001	0	0
2	40	70	6400
50	5.0	40	3200
87	1.0	20	1600
122	0.6	16	800 -
Colostrum	80	190	6400

* The vaccinia virus was measured in neutralizing skin test doses per cc.

level of about 0.03 antitoxic unit per cc. For the calf described here, the level of circulating diphtheria antitoxin was higher than this 4 months after the feeding of colostrum. This clearly indicates the important rôle that this passive immunity may play in the life of the new-born. Obviously, the persistence of immunity depends greatly on the initial titer of the colostrum and the amount fed. If the calf in this experiment had been permitted to suckle without restriction, circulating antibody concentrations might have been greater and persisted much longer. However, Hansen and Phillips (18) have recently observed that colostrum globulin is absorbed into the serum of the new-born calf only during the first 24 hours of life.

It may be estimated from the data on Lamb 1 of Mason, Dalling, and Gordon (13) that the approximate half life of immunity for lamb dysentery antitoxin acquired from colostrum is 12 to 15 days; this is similar to our data on the calf for diphtheria antitoxin. The duration of the passive immunity acquired by the feeding of colostrum in ruminants shows consider-

able variation from that of other types of passive immunity in different species. The data of Heidelberger *et al.* (19) on homologous type I pneumococcus antibody injected into a rabbit show that approximately one-half of the activity disappeared in about 36 hours, while the half life of an actively produced antibody molecule was about 2 weeks.

The difference in the duration of the passive immunity may be due more to a species difference than to the route of immunization. Two examples from the older literature may be cited. The data of Smith (20) on the

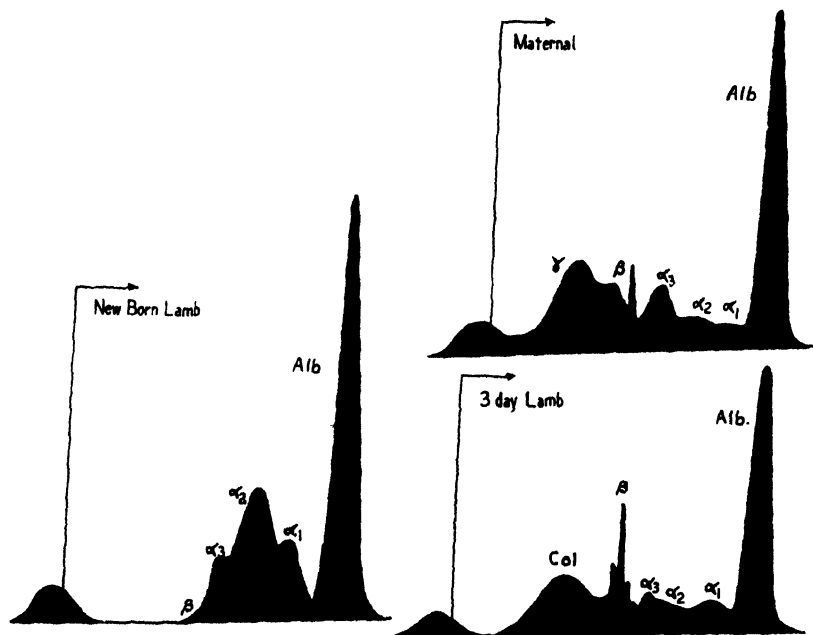


FIG. 2. Electrophoretic patterns of the descending boundaries of the sera obtained from a new-born lamb, a 3 day-old lamb, and of the mother. The runs were performed at 1° in a veronal buffer of pH 8.6 and at an ionic strength of 0.1. The serum of the new-born does not contain any slow moving globulin. This component appears only after the ingestion of colostrum.

persistence of antitetanolysin injected into a rabbit intraperitoneally or intravenously show a half life of about 1 day, in a goat of about 4 days, and for diphtheria antitoxin in man of about 3 or 4 days. On the other hand, the data of Bulloch (21) on the duration of passively acquired diphtheria antitoxin in the donkey show a half life of about 20 days.

Electrophoresis of Lamb and Sheep Sera

In Fig. 2 are shown the electrophoretic patterns obtained with the sera of a new-born lamb, of a 3 day-old lamb, and of the mother. It is quite

apparent that the serum of the new-born lamb resembles that of the calf, and is devoid of slow moving globulins which appear only after suckling has occurred. Earle (22) has already shown by Howe's salt fractionation method that the effect of colostrum ingestion on the serum proteins of young foals, kids, lambs, and pigs is similar to that found by Howe in calves.

In Table IV are presented the data for the composition and mobility of the sera shown in Fig. 2. These data were derived from photographs taken at 167 and 250 minutes. The mobility of sheep γ -globulin is quite high, and in this respect resembles the T-globulin usually present in the hyper-immune sera of the cow and the horse. Since the colostrum globulin migrates only slightly faster than the γ -globulin of the maternal serum, it

TABLE IV
Electrophoretic Analyses of Lamb and Sheep Sera

These analyses were made at 1° in veronal buffer of pH 8.5 to 8.6 and at an ionic strength of 0.1. The mobilities are $\times 10^{-5}$ sq. cm. per volt per second and are negative in sign.

Component	New-born lamb		3 day lamb		Maternal	
	u	Concentration per cent	u	Concentration per cent	u	Concentration per cent
γ			2.4*	26	2.1	26
β_2			3.6	10		
β_1	3.5	1	4.0	2	3.0	7
α_1	4.1	5	4.2	5	4.2	15
α_2	5.1	31	4.8	6	5.1	6
α_1	6.1	7	5.5	10	5.8	2
Albumin	7.6	56	6.6	41	6.7	44

* Colostrum globulin.

would be difficult in this species to distinguish between the passively acquired globulin and the adult γ -globulin. In addition to the colostrum globulin which appears in the serum of the 3 day-old lamb, the β -globulins show a marked increase, and the relative amounts of the α -globulins change considerably.

In contrast to the absence of γ -globulin from the sera of the new-born calf and lamb, Longworth, Curtis, and Pembroke (23) have recently found that in the human γ -globulin is slightly higher in the serum of the new-born than in the mother. Since it is well known that in the human there is placental transmission of antibodies, there is good correlation between the presence of γ -globulin and immunity in the new-born.

Electrophoretic examination of fetal or new-born sera of different species taken prior and subsequent to ingestion of colostrum may provide a simple

test for ascertaining whether antibodies are transferred through the placenta or the colostrum, or by both of these routes.

The authors gratefully acknowledge the technical assistance of Douglas M. Brown.

SUMMARY

1. The serum of the new-born calf does not possess any slow moving globulin (γ or T components). After ingestion of colostrum, an electrophoretic component appears in calf serum, which is identical in mobility with the lactoglobulin which has previously been demonstrated to carry immunity in bovine colostrum. Similar observations have also been made on new-born lamb serum.

2. During growth of the young calf, changes also take place in other serum components as observed by electrophoretic analysis. The γ -globulin appears some time after birth and gradually increases in concentration. The mobility and concentration of the serum albumin and α -globulin also show striking alterations.

3. Antibodies to three different antigens, two of them foreign to the species, were absorbed by the calf from colostrum. The concentration in the serum of these antibodies gradually decreased, and the time for the activity of these antibodies to drop to half their initial values was estimated as follows: for the diphtheria antitoxin about 16 days, for *Hemophilus pertussis* and vaccinia antibodies about 50 days, and for the immune component estimated by electrophoretic analysis about 20 days.

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STUDIES ON THE INHIBITORY NATURE OF 4-AMINOPTEROYLGLUTAMIC ACID

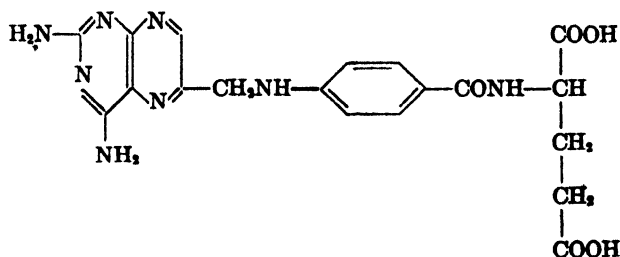
By J. J. OLESON, B. L. HUTCHINGS, AND Y. SUBBAROW

(From the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York)

(Received for publication, April 14, 1948)

The compound, N-[4-[[[(2,4-diamino-6-pteridyl)methyl]-amino]benzoyl]-glutamic acid, herein designated as 4-aminopteroylglutamic acid, has been found to be a potent antagonist of pteroylglutamic acid. Data on the inhibition of the growth of rats, chickens, and *Streptococcus faecalis* R by this compound are reported.

The structure of the compound is shown by the accompanying formula.



EXPERIMENTAL

Synthesis—The compound was prepared by the method of Seeger *et al.* (1) by condensing 2,4,5,6-tetraaminopyrimidine sulfate, 2,3-dibromopropionaldehyde, and *p*-aminobenzoylglutamic acid, and kindly supplied to us by Dr. James M. Smith, Jr., of the Calco Chemical Division, American Cyanamid Company.

Inhibition of Bacterial Growth—*Streptococcus faecalis* R was grown on the medium of Tepley and Elvehjem (2) for 18 hours in the presence of different levels of metabolite. Varying concentrations of inhibitor were added with each level of metabolite. An inhibition curve was then plotted, from which the point of half maximum inhibition was obtained. The inhibition index was calculated for each level of metabolite and is defined as the ratio of inhibitor to metabolite which will produce half maximum inhibition (3).

Rat and Chick Experiments—Weanling rats were fed the basal diet of Spicer *et al.* (4) and water *ad libitum*. The chicks were fed the basal diet of Hutchings *et al.* (5). Freshly prepared solutions of the metabolite and inhibitor in dilute sodium bicarbonate solution were fed orally or mixed in the basal diets.

Results

Inhibition of Bacterial Growth—The data on the inhibition of *Streptococcus faecalis* R and its reversal by four different metabolites are summarized in Table I. Each metabolite is capable of completely reversing the inhibition produced by 4-aminopteroylglutamic acid, but the inhibition index is not constant for different levels of the same metabolite. The fact that increasing amounts of the metabolite can reverse the effect of the inhibitors would seemingly eliminate the possibility of the antagonist affecting some enzyme not directly concerned in the metabolism of pteroylglutamic acid.

TABLE I
Inhibition of Streptococcus faecalis R with 4-Aminopteroylglutamic Acid

Metabolite	Concentration of metabolite	Inhibition index*
	γ per ml.	
Pteric acid	0.004	0.99
" "	0.04	0.32
" "	0.4	0.09
Pteroylglutamic acid	0.003	2.66
" "	0.03	0.19
" "	0.3	0.07
Pteroyl- γ -glutamylglutamic acid	0.005	4.30
" "	0.05	0.33
" "	0.5	0.10
Pteroyl- γ -glutamyl- γ -glutamylglutamic acid	0.07	0.49
	0.7	0.08
	7.0	0.007

* Ratio of the amount of inhibitor to metabolite that will produce half maximum inhibition.

The explanation of the non-constancy of the inhibition index may be that a complex is formed between the enzyme and the inhibitor that is not readily dissociable. The inhibition with 4-aminopteroylglutamic acid is not of a strictly competitive nature.

4-Aminopteroylglutamic acid is most effective as an inhibitor of the growth-promoting effect of pteroyl- γ -glutamyl- γ -glutamylglutamic acid and pteric acid. This is similar to the action of pteroylaspartic acid (3). In contrast to this, the present inhibitor is more effective against pteroylglutamic acid than against pteroyl- γ -glutamylglutamic acid.

Chick Experiments—The data from three experiments are shown in Table II. In the first experiment increasing amounts of the inhibitor were added to the basal diet containing 0.5 mg. of pteroylglutamic acid per kilo. The inhibition became apparent at the 1.0 mg. level of the antagonist and

TABLE II
Effect of 4-Aminopteroylglutamic Acid on Chicks

Group No.	Supplement per kilo basal diet	Average weight*		
		Initial day	8 days	15 days
		gm.	gm.	gm.
1	None	37 (7)	61 (8)	74 (4)
2	0.5 mg. pteroylglutamic acid	37 (7)	71 (7)	142 (7)
3	As 2 + 0.25 mg. 4-aminopteroylglutamic acid	37 (7)	70 (7)	148 (7)
4	" 2 + 0.5 " " "	37 (7)	65 (7)	137 (7)
5	" 2 + 1.0 " " "	37 (7)	66 (6)	150 (3)
6	" 2 + 2.5 " " "	37 (7)	69 (2)	108 (2)
7	" 2 + 5.0 " " "	37 (7)		
			7 days	10 days
8	0.5 mg. pteroylglutamic acid	43 (7)	87 (7)	109 (7)
9	5 mg. 4-aminopteroylglutamic acid + 0.5 mg. pteroylglutamic acid	43 (7)		
10	5 mg. 4-aminopteroylglutamic acid + 1.0 mg. pteroylglutamic acid	43 (7)		
11	5 mg. 4-aminopteroylglutamic acid + 2.5 mg. pteroylglutamic acid	43 (7)		
12	5 mg. 4-aminopteroylglutamic acid + 5.0 mg. pteroylglutamic acid	43 (7)	57 (2)	63 (2)
13	5 mg. 4-aminopteroylglutamic acid + 10.0 mg. pteroylglutamic acid	43 (7)	78 (1)	97 (1)
14	5 mg. 4-aminopteroylglutamic acid + 25.0 mg. pteroylglutamic acid	43 (7)	77 (2)	86 (2)
			7 days	21 days
15	1.0 mg. pteroylglutamic acid	36 (7)	76 (7)	188 (7)
16	4.0 mg. 4-aminopteroylglutamic acid	36 (7)		
17	4.0 " 4 " " + 4 mg. pteroylglutamic acid	36 (7)	64 (4)	160 (3)
18	4.0 mg. 4-aminopteroylglutamic acid + 12 mg. pteroylglutamic acid	36 (7)	84 (5)	245 (4)
19	4.0 mg. 4-aminopteroylglutamic acid + 24 mg. pteroylglutamic acid	36 (7)	76 (6)	212 (6)
20	4.0 mg. 4-aminopteroylglutamic acid + 48 mg. pteroylglutamic acid	36 (7)	76 (5)	194 (5)
21	4.0 mg. 4-aminopteroylglutamic acid + 96 mg. pteroylglutamic acid	36 (7)	77 (7)	188 (7)

* The figures in parentheses indicate the number alive.

all the chicks died when 5.0 mg. were added. The deaths occurred mostly in the first 3 days, indicating a very rapid interference with the normal

metabolism of the animals, as compared to simple dietary depletion of pteroylglutamic acid.

In the second experiment a completely inhibitory level of the antagonist was fed and the concentration of the metabolite was increased. It is seen that 25 mg. of pteroylglutamic acid give very little reversal of the inhibition caused by 5 mg. of the 4-amino derivative.

In the third experiment a slightly lower level of inhibitor and much higher levels of pteroylglutamic acid were fed. Complete protection against the

TABLE III
Inhibition of Rats by 4-Aminopteroylglutamic Acid

Group No.	Supplement fed orally each day	Average weight*				
		Initial day	3 days	7 days	14 days	21 days
		gm.	gm.	gm.	gm.	gm.
1	None	41 (3)	50 (3)	66 (3)	89 (3)	125 (3)
2	1 γ 4-aminopteroylglutamic acid	41 (3)	49 (3)	64 (3)	92 (3)	127 (3)
3	3 " " "	41 (3)	50 (3)	66 (2)	103 (1)	141 (1)
4	5 " " "	41 (3)	47 (3)	51 (2)	76 (2)	112 (2)
5	10 γ " "	41 (3)	43 (3)			
6	20 " " "	51 (8)	37 (3)			
7	50 " " "	41 (3)	34 (3)			
8	100 γ " "	41 (3)	31 (3)			
9	20 γ " "	52 (2)	41 (2)			
	+ 20 γ pteroylglutamic acid					
10	20 γ 4-aminopteroylglutamic acid	51 (2)	37 (2)			
	+ 100 γ pteroylglutamic acid					
11	20 γ 4-aminopteroylglutamic acid	45 (3)	46 (3)			
	+ 200 γ pteroylglutamic acid					
12	20 γ 4-aminopteroylglutamic acid	45 (3)	42 (3)			
	+ 0.2 cc. liver†					
13	20 γ 4-aminopteroylglutamic acid	45 (3)	50 (3)	41 (1)		
	+ 0.2 cc. liver + 200 γ pteroylglutamic acid					

* The figures in parentheses indicate the number alive.

† Lederle 15 unit liver, injected intramuscularly.

effects of 4 mg. per kilo of diet of the 4-amino compound was obtained with 96 mg. of pteroylglutamic acid. In the intermediate groups, a partial survival with little growth inhibition was obtained. When no pteroylglutamic acid was fed, all the chicks died in 3 to 4 days.

It seems that those chicks which survive the effects of a given level of the inhibitor in the presence of pteroylglutamic acid show little growth inhibition, and that the per cent survival varies with the level of the inhibitor used.

Rat Experiments—The inhibitor was fed to rats in graded doses, both

orally and mixed with the basal diet. Reversal of the inhibition was attempted by giving large amounts of pteroylglutamic acid. The data are summarized in Tables III and IV.

TABLE IV
Inhibition of Rats by 4-Aminopteroylglutamic Acid

Group No.	Supplement to basal diet	Average weight*				
		Initial day	3 days	7 days	14 days	21 days
		gm.	gm.	gm.	gm.	gm.
14		44 (4)	52 (4)	66 (4)	94 (4)	124 (4)
15	0.25 mg. 4-aminopteroylglutamic acid	41 (5)	42 (5)	51 (5)	74 (5)	100 (4)
16	0.5 mg. 4-aminopteroylglutamic acid	42 (9)	46 (9)	54 (9)	66 (7)	80 (5)
17	1.0 mg. 4-aminopteroylglutamic acid	42 (10)	39 (9)	61 (1)		
18	2.0 mg. 4-aminopteroylglutamic acid	43 (7)	35 (6)			
19	5.0 mg. 4-aminopteroylglutamic acid	41 (3)	33 (3)			
20	1.0 mg. 4-aminopteroylglutamic + 1 mg. pteroylglutamic acid	44 (4)	37 (4)			
21	1.0 mg. 4-aminopteroylglutamic acid + 5 mg. pteroylglutamic acid	44 (4)	39 (4)	40 (1)		
22	1.0 mg. 4-aminopteroylglutamic acid + 10 mg. pteroylglutamic acid	44 (4)	44 (4)	38 (3)		
23	1.0 mg. 4-aminopteroylglutamic acid + 15 mg. pteroylglutamic acid	42 (2)	46 (2)			
24	1.0 mg. 4-aminopteroylglutamic acid + 20 mg. pteroylglutamic acid	41 (6)	45 (6)	45 (1)	57 (4)	76 (4)
25	1.0 mg. 4-aminopteroylglutamic acid + 25 mg. pteroylglutamic acid	44 (4)	41 (4)	60 (4)	75 (4)	95 (3)
26	1.0 mg. 4-aminopteroylglutamic acid + 30 mg. pteroylglutamic acid	40 (4)	47 (4)	53 (4)	69 (4)	89 (4)
27	1.0 mg. 4-aminopteroylglutamic acid + 40 mg. pteroylglutamic acid	40 (4)	48 (4)	51 (4)	65 (4)	84 (4)

* The figures in parentheses indicate the number alive.

It can be seen that the compound is extremely toxic for the rat, since as little as 10 γ orally per day or 1 mg. per kilo of diet kills the animals in about 3 days.

The symptoms produced in the rat by feeding this inhibitor consist of a severe diarrhea, resulting in extreme dehydration, weight loss, porphyrin-stained whiskers, and hemoconcentration. Prior to death there are signs of nervous involvement and anoxia. Preliminary histological observations show extensive tissue changes and a very hypoplastic bone marrow.

As in the case of the chick, very high levels of pteroylglutamic acid are required to obtain even a partial reversal on the rats. Since the amount of inhibitor required to produce a severe inhibition is so near the amount which kills the animal, an exact neutralization ratio is difficult to determine. As in the case of the chicken, it appears to be an inhibition which is reversed with considerable difficulty.

The question of whether the rapid death of the chicks and rats after administration of the inhibitor is due to a pteroylglutamic acid deficiency *per se* or to other pharmacological properties of the compound is not clearly answered by the above data. The fact that large amounts of pteroylglutamic acid can prevent the onset of the symptoms would seem to favor the former possibility.

DISCUSSION

These results make it evident that 4-aminopteroylglutamic acid is a powerful antagonist for pteroylglutamic acid and related metabolites.

With *Streptococcus faecalis* R, the inhibition index was different for the different metabolites. Since a constant index for any given metabolite was not obtained, this inhibition is not a classical example of competitive inhibitions. A slightly different inhibition pattern for *Streptococcus faecalis* R was obtained with this inhibitor as compared to results obtained previously with pteroylaspartic acid (3).

For rats and chickens the inhibition would appear to be competitive, but only in the general sense that very high doses of metabolite are required to remove the toxic manifestations of the inhibitor. The quantity of inhibitor needed to produce effective inhibitions of the animals is so nearly that which rapidly kills the animals that the working range is very narrow.

Blood studies indicate that a severe anemia can be produced by feeding this compound and that the anemia can be prevented or cured by the administration of pteroylglutamic acid.¹

It would seem necessary to handle this compound with the same precautions used for other toxic substances.

SUMMARY

4-Aminopteroylglutamic acid was found to be an antagonist for pteroylglutamic acid for *Streptococcus faecalis* R, rats, and chickens.

The inhibition is reversible but is not of a strictly competitive nature.

¹ Unpublished data.

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A CHEMICAL DETERMINATION OF STREPTOMYCIN IN BODY TISSUES AND URINE

BY VIOLA C. JELINEK AND GEORGE E. BOXER

(From the Research Laboratories of Merck and Company, Inc., Rahway, New Jersey)

(Received for publication, May 4, 1948)

An adequate dosage for chemotherapeutic agents is generally established on the basis of the concentration of the particular drug in the blood. It is, however, a well recognized fact that for some antibiotics in a number of infections the site of the therapeutic action may not be the blood stream itself, but rather the affected tissues and their interstitial fluid. Information on the concentration of streptomycin in the tissues will be of particular interest in the therapy of the non-hematogenous forms of tuberculosis.

In previous papers, we have described chemical methods for the assay of streptomycin in clinical preparations, broth, urine, blood, and spinal fluid. These methods were based on the formation of maltol from streptomycin by heating with alkali (1) or on the formation of a fluorescent hydrazone of streptomycin (2). The fluorometric method affords a high degree of sensitivity, sufficient to measure the amounts of streptomycin present in blood and spinal fluid. This procedure with its accuracy and reproducibility permitted the demonstration of the detailed and predictable relationship between the amount of the drug injected and the blood levels obtained (3). A colorimetric method based on an analogous reaction of the aldehyde group of streptomycin, but of somewhat less sensitivity than the fluorometric assay, has been independently reported by Marshall, Blanchard, and Buhle (4).

The distribution of streptomycin in the tissues of experimental animals has been determined by means of microbiological assay methods (5, 6) which are, however, subject to considerable inherent error. These data indicated that the quantities of streptomycin in the tissues following injection of therapeutic doses are of the order of magnitude of 1 to 20 γ per gm. of tissue. Since the fluorometric procedure is sufficiently sensitive to measure such amounts, it has been adapted for the assay of streptomycin in tissues.

The quantities of streptomycin excreted in the urine are usually large enough to be measured by a method based on maltol formation (1). For urinary clearance studies, however, it is desirable to measure the concentration of the drug in the blood and urine by a method based on the same chemical reaction, and the fluorometric procedure was thus extended for the assay of streptomycin in urine. Identical values were obtained with both methods, maltol formation and the fluorometric procedure.

In the fluorometric assay of streptomycin in blood a very small and essentially constant blank was obtained with the protein-free blood filtrates (2). Appreciable and variable blank values were encountered when the fluorometric method was applied to deproteinized filtrates from normal tissues. Usually the blank value for urine is nearly that of the reagent blank, since the urine is greatly diluted for assay; however, if very small quantities of streptomycin are to be determined in undiluted urine, the blank value becomes significant. These blanks can, however, be accurately determined by selectively destroying the streptomycin without altering the amounts of the interfering substances present. Either acid or alkali destroys streptomycin; the choice of pH depends on the effect on the interfering substances. Heating in dilute acid will selectively destroy streptomycin in protein-free extracts of liver, brain, lung, heart, and spleen without changing the quantity of interfering substances present. Acid destruction was previously used in the assay of streptomycin in broth by the maltol procedure (1). In urines, heating with acid not only destroyed the streptomycin but also liberated additional interfering material. A satisfactory urine blank was obtained by alkali heat treatment which converts streptomycin into maltol, not reactive in the fluorometric procedure, while the amount of interfering material was not changed.

The fluorometric assay of streptomycin in liver was somewhat more involved. The deproteinized extracts of livers from normal well nourished animals contain large quantities of glycogen. Although glycogen does not react with acridyl hydrazine and is not inherently fluorescent, it will give spurious readings in the fluorophotometer due to the scattering of light. Removal of the glycogen from the protein-free extract was, of course, easily obtained by precipitation with an equal volume of ethyl alcohol, but it was necessary to remove the bulk of the alcohol, since the presence of appreciable quantities of ethanol seriously decreased the sensitivity of the assay.

Wide variations, from 0 to 3000 γ per ml., of the concentration of streptomycin in urine will be encountered in excretion studies. The dilution of the urine to the proper concentration for the fluorometric assay, 1 to 20 γ , may not always be accurately predicted, and a determination may be out of range. Simple dilution of the final solution is not feasible, since the fluorescence is critically dependent on the acidity. The acridyl hydrazone of streptomycin, in addition to being fluorescent, has a yellow color in higher concentrations. This color was used to extend the usefulness of the hydrazine method to a range from 1 to 500 γ of streptomycin. Thus, an assay which is out of range for the measurement of fluorescence can still be completed by determining the light absorption at a wave-length of 440 m μ .

When the calibration curve of the fluorometric method was checked occasionally, it was noted that the curve had a definite lower slope on hot

days. In order to demonstrate that the intensity of the fluorescence of the acridyl hydrazone of streptomycin was dependent on temperature, fluorophotometric readings were taken on a solution of acridyl hydrazone from 20 γ of streptomycin while increasing and decreasing the temperature from 22–44°. In Fig. 1 the results of this experiment are represented graphically. The arrows indicate whether the reading was obtained on the heating or cooling phase of the experiment. From the smooth curve it is apparent that variations due to small, ordinary room temperature fluctuations will be significant only for the most accurate work. When the temperature is markedly different from that at which the calibration was performed, a percentage correction factor should be applied which is readily obtained from Fig. 1. Alternatively, one can construct a set of calibration curves

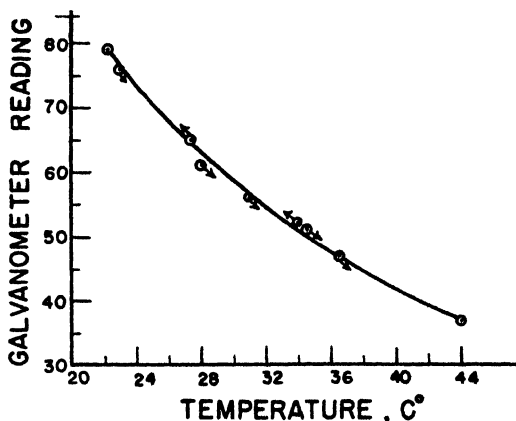


FIG. 1. Influence of temperature on the fluorescence of the acridyl hydrazone of streptomycin.

at several useful temperatures. In any case, at a given temperature the fluorescence was still strictly proportional to the streptomycin present.

EXPERIMENTAL

Reagents—

Reagents as described for the determination of streptomycin in blood (2).

Isoamyl alcohol (7 volumes)-benzene (3 volumes) mixture.

4 N NaOH.

4 N HCl.

Determination in Urine—The quantity of streptomycin in urine was measured by difference, the amount of interference being subtracted from the total amount of fluorescent material. For the "total" determination

an aliquot of urine containing less than 20 γ of streptomycin was diluted to 14 ml., 1 ml. of 4 N HCl and 3 ml. of the acridyl hydrazine reagent were added, and the procedure for the determination of streptomycin in aqueous solution was followed (2). 4 N HCl was substituted for 3 N HCl in the procedure for an aqueous solution to insure adequate acidity in the presence of the urinary buffers. The aliquot of urine used for a determination was usually considerably less than the equivalent of 0.1 ml. of undiluted urine. With more than 0.5 ml. of undiluted urine, recoveries were low, the blanks were excessively high, and the galvanometer readings were unstable, drifting upward. None of these difficulties were ever encountered if 0.5 ml. or less of the undiluted urine was used.

TABLE I
Experimental Basis for Differential Assay of Streptomycin in Urine

Species	Volume of urine ml.	Galvanometer reading of urine blank	
		Directly	After heating with alkali
Rabbit	0.2	14.0	14.2
"	0.5	27.0	26.5
"	1.0	43.5	43.3
Dog	0.2	16.2	16.0
"	0.5	28.8	29.5
"	1.0	49.0	51.0
Man	0.5	9.0	8.2
"	0.5	12.8	13.0
"	0.5	9.0	8.8
"	0.5	15.5	16.0
"	0.5	13.8	12.0
"	0.2	10.0	10.3

The "urine blank" was obtained with an equal aliquot diluted to 5 ml., and the streptomycin was converted to maltol by adding 1 ml. of 3 N NaOH and immersing the tube in a boiling water bath for 3 minutes. The tube was cooled, and 7 ml. of water were added, followed by 2 ml. of 4 N HCl and 3 ml. of hydrazino-acridine reagent. The subsequent reaction and the extraction were as usual (2).

Table I contains representative results which establish the essential requirement for a useful blank; namely, that the process which destroyed the streptomycin in urine does not alter the amount of interfering material.

Since clinical experiments frequently require storage of urine samples prior to assay, the stability of streptomycin added to normal urine kept at refrigerator temperature was determined daily for a week. Both the

"total" determination and the amount of interference remained constant for this period.

Determination in Tissue—The initial steps of homogenization and deproteinization were the same for all the tissues, the entire procedure being accomplished in the Waring blender. 5 gm. of tissue were minced, suspended in water to a volume of 80 ml., and homogenized in the blender for 3 to 5 minutes. With the blender still in operation, the homogenate was deproteinized with 10 ml. of 10 per cent $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, followed after 1 minute with 10 ml. of 0.5 N NaOH. Mixing was continued for about $\frac{1}{2}$ minute before filtering. The quantities suggested can be efficiently manipulated in the macro container of the blender; smaller quantities can be processed either in the micro container or by manual grinding and deproteinization.

TABLE II
Experimental Basis for Differential Assay of Streptomycin in Tissues

	Galvanometer reading of tissue blank	
	Directly	After heating with acid
Heart	9.0	8.8
Brain	11.0	10.2
"	12.3	12.0
"	10.2	9.3
Lung	9.8	8.8
"	8.2	7.5
"	11.8	10.3
Spleen	14.0	15.3

(a) *Lung, Brain, Heart, Spleen*—Two aliquots of the protein-free filtrate, containing 0.5 gm. of tissue per 10 ml., were used in a differential method similar to that for urine. The "total" value was determined in a 10 ml. aliquot by adding 4 ml. of water, 1 ml. of 4 N HCl, and 3 ml. of the acridine reagent, and continuing the procedure for the determination of streptomycin in aqueous solution (2).

To obtain a "tissue blank," streptomycin was destroyed in the second aliquot by heat treatment with acid. To a 10 ml. aliquot were added 3 ml. of 4 N HCl, and the tube was immersed in boiling water for 5 minutes. After cooling, 2 ml. of 4 N NaOH and 3 ml. of hydrazino-acridine reagent were added and the procedure was completed as usual (2).

Evidence in Table II is presented to show that the amount of interfering material is not altered when streptomycin is destroyed in tissue extracts by heating with acid. Representative values obtained with rabbit tissues are recorded.

(b) *Liver*—The procedure for liver is essentially the same as that described for the other tissues, with the exception of additional steps for the removal of glycogen. An equal volume of absolute ethanol was added to 25 to 30 ml. of protein-free liver filtrate and the glycogen precipitate was permitted to settle for 15 to 30 minutes. After centrifuging, the bulk of the ethanol was removed from two 20 ml. aliquots (20 ml. $\approx$ 0.5 gm. of tissue) of the supernatant by extracting once with 20 ml. of isoamyl alcohol-benzene mixture for 10 seconds. The water phase, 8.5 ml., was collected in a 25 ml. glass-stoppered graduate. For the "total" determination the volume was brought to 14 ml. with water, and 1 ml. of 4 N HCl and 3 ml. of the acridine reagent were added. The reaction and separation procedures were as usual (2), except for the addition of 4 ml. of water before the separation with benzyl alcohol was begun. The presence of a small amount of residual ethanol causes a decrease of volume of the aqueous phase in the separation procedure, making this addition of water necessary to obtain the proper final volume of 15 to 16 ml.

The water phase from the other aliquot was diluted to 10 ml. with water, 3 ml. of 4 N HCl were added, and the streptomycin was destroyed by heating in a water bath at 80° for 15 minutes. The usual procedure at 100° would cause excessive boiling because of the small amount of residual alcohol. Subsequently 2 ml. of 4 N NaOH and 3 ml. of the acridine reagent were added, followed by the reaction and separation as usual, including the addition of 4 ml. of water, as in the corresponding "total" determination.

The calibration curve obtained with streptomycin in 50 per cent ethanol with subsequent alcohol removal is slightly less sensitive than that obtained directly in the absence of alcohol. Thus, when this procedure is used for the determination of streptomycin in liver, a calibration curve involving the extraction of ethanol with isoamyl alcohol-benzene must be established.

Results

Analytical recoveries of streptomycin added to the normal urine of man and dog were determined on a number of samples. The recoveries were quantitative within the experimental errors of the fluorometric method, provided 0.1 ml. or less of undiluted urine was used per test. In six experiments on human urine, to which from 20 to 100 γ of streptomycin per ml. had been added, 99 ± 9 per cent were recovered when 0.2 ml. of urine per assay was used. The recovery obtained with twelve samples of human urine containing from 2 to 50 γ of the drug per ml. was 95 ± 6 per cent when 0.5 ml. was used per test. In similar experiments with ten samples of dog urines containing from 25 to 100 γ per ml. there was a recovery of 93 ± 5 per cent of the added drug when 0.2 ml. of urine was used, while 87 ± 7 per cent was recovered in nine cases in which 0.5 ml. of undiluted dog urine was analyzed containing from 10 to 40 γ per ml.

The fluorometric method is sufficiently sensitive to determine 1 γ of streptomycin per test (2). Thus urine containing 10 γ per ml. can be assayed with 0.1 ml. of undiluted urine for the determination with no correction involved for recovery. In the rare cases in which smaller concentrations are to be measured, correspondingly larger volumes must be used, and appropriate corrections made from the recovery data just presented. If 0.5 ml. of the urine is used per test, the lower limit is 2 γ per ml.

In Table III typical values on urines from patients and dogs receiving streptomycin intramuscularly are reported. The results from three methods of assay are compared: the fluorometric method, the colorimetric determination of the acridyl hydrazone of streptomycin, and the assay based on

TABLE III

Determination of Streptomycin in Urine after Intramuscular Injection; Comparison of Various Chemical Methods

Urine,	Streptomycin determined by		
	Maltol method, phenol reagent	Acridyl hydrazine method	
		Fluorometric	Colorimetric
	γ per ml.	γ per ml.	γ per ml.
Dog	178	160	
"	385	413	
"	799	832	
"	216	225	
"	59	66	
"	127	149	
Man	823	704	748
"	505	482	531
"	755	818	803
"	1018	960	913
"	539	610	593

maltol formation. The agreement between the three methods was satisfactory, and, if the quantities of streptomycin are relatively large, any of these methods will give the desired results. For clearance experiments a method based on acridyl hydrazone formation is preferable, since the determination in both urine and blood is based on the same reaction. The fluorometric is, of course, more sensitive than the maltol method. Furthermore, the acridyl hydrazone method is not influenced by contamination of the urine with feces, which seriously interferes in the maltol method.

Recovery experiments of streptomycin added to various tissues of several species are summarized in Table IV.

In these experiments the drug was of necessity added to the homogenate of the tissue and the essentially complete recoveries indicate that none of

the streptomycin was adsorbed during the deproteinization or lost during subsequent operation. In the case of the parenteral administration of streptomycin the possibility cannot be ruled out that some of the drug

TABLE IV
Analytical Recovery of Streptomycin Added to Tissue

Species	Tissue	No. of experiments	Amount of streptomycin added per 0.5 gm. tissue	Per cent recovery and standard deviation
			γ	
Dog	Liver	5	11.1-19.9	103 $\pm$ 14
"	Lung	6	1.4-13.9	98 $\pm$ 8
"	Brain	9	1.4-19.5	96 $\pm$ 10
"	Spleen	9	1.4-19.5	96 $\pm$ 8
Rabbit	Liver	15	1.4-28.0	97 $\pm$ 11
"	Lung	7	2.8-20.9	99 $\pm$ 6
"	Brain	6	2.8-20.9	99 $\pm$ 6
"	Spleen	1	10.7	95
"	Heart	1	10.0	94
Cat	Lung	5	2.8-13.9	110 $\pm$ 17
"	Brain	9	1.4-19.5	96 $\pm$ 10
"	Spleen	1	2.8	93

TABLE V
Determination of Streptomycin in Organs of Rabbits after Intramuscular Injection of Total of 60,000 γ per Kilo of Streptomycin, Injected in Three Equal Doses at 2 Hour Intervals

Streptomycin	Rabbit A. Killed 1 hr. after last injection	Rabbit B. Killed 24 hrs. after last injection
	γ per ml.	γ per ml.
Plasma	75.5	19.1
	γ per gm.	γ per gm.
Liver	11.2	3.6
Lung	23.2	0.0
Heart	16.2	1.8
Spleen	10.2	<1.0
Brain	2.8	1.4

might be bound to the insoluble particles of the cells and could therefore not be released on homogenization into the solution.

An example of the application of the fluorometric method to the determination of streptomycin in tissues of animals receiving the drug intramuscularly is reported in Table V.

Thus it is demonstrated that streptomycin administered to the living

animal can be analytically determined in the tissues by the method described. Since the relatively small concentrations of streptomycin found in the tissues could be entirely due to the blood content of the organs, interpretation of the results must be deferred to a more detailed study.

SUMMARY

A fluorometric method for the determination of streptomycin in urine and tissue has been described.

The lower limit of sensitivity is 2 γ per ml. of urine and 2 γ per gm. of tissue.

Data on the analytical recoveries of streptomycin added to urine and to lung, liver, brain, heart, and spleen are presented.

The utility of this method for the determination of streptomycin in urine and tissues following parenteral administration has been demonstrated.

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INVESTIGATIONS ON THE MUNG BEAN (*PHASEOLUS AUREUS* ROXBURGH)

I. THE DETERMINATION OF EIGHTEEN AMINO ACIDS IN THE MUNG BEAN HYDROLYSATE BY CHEMICAL AND MICROBIOLOGICAL METHODS*

BY W. EDWARD BELTON AND CECILE A. HOOVER

(From the Department of Chemistry, Tuskegee Institute, Alabama)

(Received for publication, February 20, 1948)

The mung bean, *Phaseolus aureus* Roxburgh, has been utilized as a source of protein in chick diets. Elementary feeding experiments conducted at Tuskegee Institute and elsewhere indicated that the addition of supplementary amino acids is required to increase the effectiveness of the mung bean as a source of complete protein in the diet. Since these amino acids and the amounts in which they must be added were not known, the present study was undertaken to determine the amino acid composition of the total protein of the mung bean.

Previous work on the analysis of the mung bean has been limited to an elementary chemical analysis by Heller (1) and determinations of the nitrogen distribution by Heller (2) and Johns and Waterman (3). In addition, the tyrosine and tryptophan contents of the α - and β -globulins of the mung bean have been determined by Jones (4), using colorimetric methods.

EXPERIMENTAL

Preparation of Sample—All samples of the mung bean used in these experiments were prepared from beans grown on experimental plots. The beans were ground and the fat extracted with ether in a Soxhlet apparatus for 18 hours.

From previous experiments undertaken to determine complete hydrolysis, it was found that autoclaving a 5 gm. sample with 60 ml. of 10 per cent hydrochloric acid at 15 pounds pressure for 5 hours gave best results. For tyrosine and tryptophan, alkaline hydrolysis with 50 ml. of 5 N sodium hydroxide in an autoclave at 15 pounds pressure for 5 hours was used. In all subsequent work, these conditions of hydrolysis were employed.

The total nitrogen was determined by the micro-Kjeldahl method and found to be 3.73 per cent.

* This work was aided by a grant from Swift and Company to the Carver Research Foundation. The material in this paper was taken in part from a thesis submitted by C. A. Hoover in partial fulfillment of the requirements for the degree of Master of Science in Chemistry, Tuskegee Institute, Alabama.

Colorimetric Assay—Quantitative work on colorimetric analysis included assays of arginine, methionine, tyrosine, tryptophan, phenylalanine, and valine. The acid hydrolysate was used for all analyses except those of tyrosine and tryptophan. Varying concentrations of the standard solutions¹ and hydrolysate were prepared. From readings obtained on a Cenco Sheard-Sanford photometer, the standard curves were plotted on Cenco semilogarithmic paper. Solutions of the hydrolysate were treated in a similar manner and the values obtained were interpolated on the standard curve for the corresponding amino acid. From these, percent-

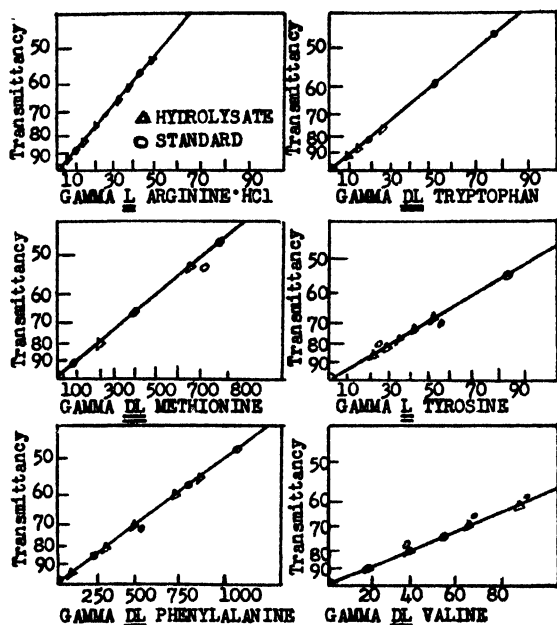


FIG. 1. Colorimetric assay

ages of the amino acids present in the hydrolysate were obtained. The data for these analyses are summarized in Fig. 1.

Microbiological Assay—The assay techniques employed in the microbiological analysis were those of Dunn *et al.* (5-7) for *Leuconostoc mesenteroides* P-60 and Baumgarten *et al.* (8) for *Lactobacillus arabinosus* 17-5 and *Streptococcus faecalis*. Experiments were run in quadruplicate with 9 ml. of the basal medium made up to a final volume of 10 ml. by the addition of varying ratios of distilled water and the standard solution or the

¹ C.P. grade amino acids were used in all assays. The folic acid, under the trade name Folvite, was purchased from the Lederle Laboratories Division, American Cyanamid Company.

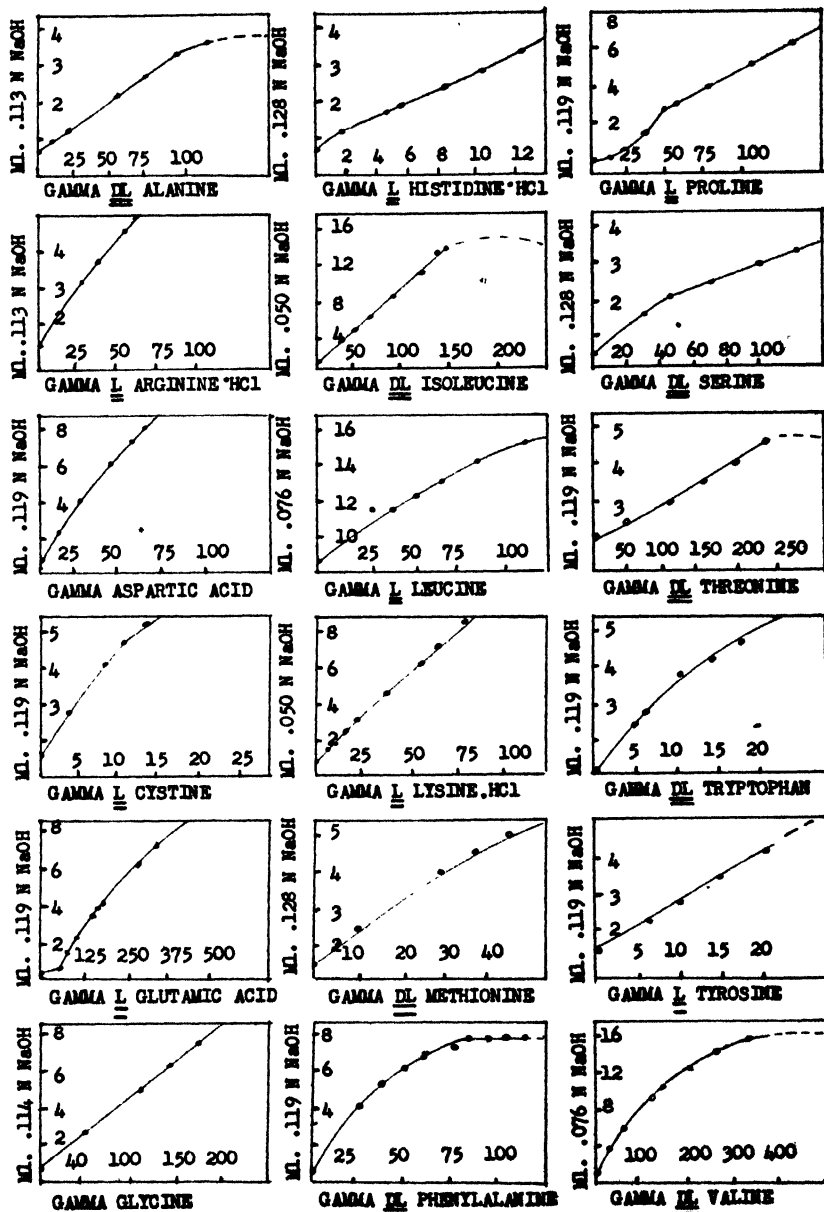


Fig. 2. Microbiological assay

hydrolysate. The tubes were autoclaved 15 minutes at 15 pounds pressure and incubated 72 hours at 35–37°. Titrations were made electrometrically

with standard base to pH 7 from a micro burette. Standard curves for microbiological assays of the amino acids are shown in Fig. 2.

Recovery Experiments—To determine the validity of assay results, recovery experiments were conducted on the mung bean hydrolysate with added amounts of arginine, isoleucine, lysine, methionine, phenylalanine, threonine, and tryptophan. The results are summarized in Table I.

TABLE I
Results of Recovery Experiments on Amino Acid Test Mixture

Amino acid	Titration volume per tube	Amino acid		Recovery
		Added	Found	
	ml.	γ	γ	per cent
Arginine	1.86	8.0	8.0	100
	2.90	20.0	19.5	98
	4.34	40.0	40.0	100
Isoleucine	2.90	26.0	26.0	100
	4.24	52.0	52.0	100
	5.36	77.0	75.0	97
Lysine	6.36	103.0	102.0	99
	1.56	4.0	4.0	100
	1.74	10.0	10.0	100
	2.10	20.0	20.0	100
	2.62	30.0	30.0	100
	3.40	40.0	41.0	102
Methionine	3.76	4.3	4.6	105
	4.70	14.0	13.0	98
	5.78	22.0	23.5	106
	6.60	32.0	31.5	98
	7.74	43.5	43.5	100
Phenylalanine	3.18	8.8	8.9	101
	5.85	44.0	44.0	100
	6.76	66.0	66.0	100
Threonine	1.30	20.0	20.0	100
	2.12	60.0	60.0	100
	2.72	80.0	80.0	100
Tryptophan	2.10	2.1	2.2	104
	2.10	4.1	4.1	100
	3.80	6.2	6.2	100
	4.70	8.2	8.4	102

Microbiological, colorimetric, and Van Slyke nitrogen distribution values for eighteen amino acids were summarized and compared in Table II.

DISCUSSION

The values reported for the nitrogen distribution experiments have been checked against similar values reported by previous investigators. The

arginine value obtained in this investigation, 11.10 per cent, is considerably lower than the value reported by Heller; however, the histidine and cystine values, 6.73 and 1.66 per cent, respectively, agree closely with values reported by Heller, 6.76 and 1.62 per cent. The value reported for lysine is lower than that of Heller. However, it should be pointed out that the lysine value is obtained by difference; hence, any discrepancy in the analysis of the total basic fraction would be reflected in the lysine value.

TABLE II

Comparison of Amino Acid Assay Values on Mung Bean with Other Work Previously Reported in Literature

Amino acid	Microbiological analysis	Colorimetric analysis	Chemical analysis	
			This work	Heller (2)*
	per cent	per cent	per cent	per cent
Alanine	0.81			
Arginine	2.56	2.61	2.56	3.15
Aspartic acid	1.33			
Cystine	0.29		0.38	0.37
Glutamic acid	11.69			
Glycine	9.07			
Histidine	1.52		1.56	1.57
Isoleucine	0.33			
Leucine	9.12			
Lysine	3.81		2.58	2.98
Methionine	0.62	0.62		
Phenylalanine	2.76	2.60		
Proline	5.27			
Serine	2.85			
Threonine	1.88			
Tryptophan	0.63	0.60		
Tyrosine	1.37	1.54		
Valine	6.16	6.20		

* The values in the last column were recalculated in terms of per cent amino acid in the protein for the sake of comparison.

Whereas the quantitative assay involved the actual determination of eighteen amino acids, tests indicate the qualitative presence of hydroxyproline and cysteine which cannot be determined microbiologically.

By using the microbiological assay method, smooth standard curves, recovery of amino acids within the limit of experimental error, and identical results at various levels of the hydrolysate were established in assays with *Leuconostoc mesenteroides* P-60, *Lactobacillus arabinosus* 17-5, and *Streptococcus faecalis*.

Eighteen amino acids in the mung bean were determined microbio-

logically. Alanine, histidine, lysine, methionine, and serine were determined with *Streptococcus faecalis*; aspartic acid, glycine, leucine, phenylalanine, proline, tyrosine, tryptophan, threonine, and valine were determined with *Leuconostoc mesenteroides* P-60, and isoleucine was determined with *Lactobacillus arabinosus* 17-5.

The microbiological value for lysine, 3.81 per cent, was considerably higher than the value obtained by the Van Slyke method, 2.58 per cent. However, this value is in agreement with the value reported by Heller. The values obtained for arginine by the Van Slyke method, 2.56 per cent, and the colorimetric method, 2.61 per cent, compare favorably with that obtained by the microbiological assay method, 2.56 per cent. The microbiological values for histidine and cystine, 1.52 and 0.29 per cent, respectively, check closely with values obtained by the nitrogen distribution method, 1.56 and 0.38 per cent, and those reported by Heller, 1.57 and 0.37 per cent.

In addition to the colorimetric value for arginine (discussed earlier), colorimetric assays have been conducted for methionine, phenylalanine, tryptophan, tyrosine, and valine. These values were found to be in excellent agreement with those obtained by the microbiological assay method (Table II).

Since the values obtained for the five monoamino acids compared favorably, it is felt that the values reported, for alanine 0.81 per cent, aspartic acid 1.33 per cent, glutamic acid 11.69 per cent, glycine 9.07 per cent, isoleucine 0.33 per cent, leucine 9.12 per cent, proline 5.27 per cent, serine 2.85 per cent, and threonine 1.88 per cent, are accurate within the experimental limits of the microbiological assay method.

According to Stokes and Gunness (9), the synthetic racemate of each amino acid was found to be exactly one-half as active as the L isomer, indicating that the D, or unnatural isomer, was inactive. Also, identical standard curves were obtained with the L and DL forms, when twice as much of the latter was used. Hence, either form can be used as a standard for assay purposes.

SUMMARY

The amino acid composition of the total protein of the mung bean, *Phaseolus aureus* Roxburgh, has been determined by chemical and microbiological assay methods.

1. Data are reported on the quantitative colorimetric assay of arginine, methionine, valine, tyrosine, tryptophan, and phenylalanine.

2. Evidence has been cited to substantiate the validity of the values reported.

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THE EXTINCTION COEFFICIENTS OF THE REDUCED BAND OF PYRIDINE NUCLEOTIDES

By B. L. HORECKER AND ARTHUR KORNBERG

(From the Division of Physiology, National Institute of Health, Bethesda, Maryland)

(Received for publication, May 4, 1948)

Warburg and Christian (1) observed that the reduced forms of diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) have absorption bands with maxima at 340 m μ , whereas the oxidized forms have no absorption at this wave-length. Application of this observation to the quantitative determination of the pyridine nucleotides and of substrates which can be brought into stoichiometric reaction with them has been hampered by the lack of reliable extinction coefficients for these substances. The published values for DPN, recently reviewed by Drabkin (2), vary from 4.78×10^6 to 6.28×10^6 sq. cm. $\times$ mole⁻¹. In the case of those values which were determined by calculation from the absorption of a given quantity of nucleotide which was assumed to be pure, it may be presumed that the purest samples yielded the highest values, although no good criterion of purity is available. The highest value thus far reported, 6.28×10^6 , was obtained by Ohlmeyer (3) for a sample of isolated reduced DPN. In the case of TPN there is considerably less information, although the molecular extinction coefficient has been reported to be the same as for DPN (4).

Precise values for the extinction coefficients can be determined with pyridine nucleotide preparations which are not necessarily pure by the use of pure substrates in reactions which are essentially complete. Thus, with an excess of pyridine nucleotide over substrate under suitable conditions the change in absorption would be due to the reaction of a quantity of nucleotide equivalent to the added substrate.

Such determinations have been made with pyruvic acid, acetaldehyde, and isocitric acid. For the reactions,



the equilibrium constants have been reported as 1.7×10^4 (5), 1.4×10^3 (6), and 7.7×10^3 (7), respectively. In the case of acetaldehyde, with the smallest constant, the reaction would proceed to about 99.9 per cent of completion with a 2-fold excess of DPNH_2 . The extinction coefficients obtained with both DPN and TPN in these reactions agree within 2 per cent and confirm Ohlmeyer's value.

EXPERIMENTAL

Absorption Measurements

The absorption measurements were made with a Beckman model DU quartz spectrophotometer, with a slit width of 1.6 $m\mu$ at 340 $m\mu$. 1.00 cm. cells with Corex D windows were used throughout. The results are reported as optical density ($\log_{10} I_0/I$).

The extinction coefficient, ϵ , was obtained from the relation $\log_{10} I_0/I = \epsilon cl$ where the concentration, c , was expressed in moles per cc. and the length, l , in cm.

Pyridine Nucleotides

Reduced Diphosphopyridine Nucleotide—DPN (purity 0.70) prepared by the method of Williamson and Green (8) was reduced and isolated according to Ohlmeyer (3). From the change in density at 340 $m\mu$ on oxidation with excess pyruvate in the presence of lactic dehydrogenase, the purity was determined to be 0.50. The concentration of $DPNH_2$ declined slowly over a period of several months.

Triphosphopyridine Nucleotide—This was obtained from liver by a modification of the method of Warburg and Christian (1).¹ The TPN content was determined spectrophotometrically by reduction with excess isocitrate in the test system described below. The preparation had a purity of 0.55.

Enzymes

Lactic Dehydrogenase—A purified preparation was obtained by repeated ammonium sulfate fractionation of an extract of rabbit muscle.²

Isocitric Dehydrogenase—This was prepared according to Ochoa and Weisz-Tabori (9) by extraction of pig heart acetone powder with 0.1 M phosphate buffer at pH 7.3, followed by dialysis against running tap water.

Alcohol Dehydrogenase—An acetone powder was prepared from washed dried brewers' yeast according to Steps I and II as described by Negelein and Wulff (6). With their spectrophotometric test the preparation was found to have a purity of 0.025.

Substrates and Test Systems

Pyruvic Acid—Eastman pyruvic acid was freshly distilled *in vacuo* for each experiment. Fractions were collected at 18 mm. and 67.5–68.5° in one distillation and at 14 mm. and 61.5–62.5° in a second. Samples were weighed and dissolved immediately after distillation. Dilute samples were prepared as required from stock solutions containing about 1 mg. per cc.

¹ Unpublished procedure of Warburg and Christian furnished by Dr. Erwin Haas.

² Unpublished method.

For the oxidation of DPNH_2 by pyruvic acid, the test systems contained 0.132 micromole of DPNH_2 and 0.27 mg. of the lactic dehydrogenase preparation in 1.41 cc. of 0.035 M phosphate buffer at pH 7.4. After measurement of the density, 0.05 to 0.10 cc. of the pyruvic acid solution was added and the density observed at 340 $m\mu$ until a constant value was reached. This required 2 to 5 minutes.

Although lactic dehydrogenase from animal tissue has been described as specific for DPN (10), it has recently been demonstrated (11) that TPNH_2 will undergo oxidation by pyruvate in the presence of the enzyme, although at a much slower rate than DPNH_2 . The oxidation of TPNH_2 was accomplished by the addition of pyruvate after the TPN had been reduced by isocitrate, as described in the following section.

Isocitric Acid—Solutions were prepared from weighed samples of recrystallized *dl*-isocitric acid and neutralized before use. Since only the naturally occurring *d* isomer (12) reacts in this test,³ the concentration used in the calculations was based on one-half the total isocitrate added.

The test system for the reduction of TPN consisted of 0.14 micromole of TPN, 0.25 mg. of the isocitric dehydrogenase preparation, MnCl_2 to a final concentration of 7×10^{-6} M, and veronal buffer of pH 7.3 (13) to a final concentration of 0.07 M, in a total volume of 1.40 cc. The reaction mixture also contained 5.4 mg. of the lactic dehydrogenase preparation to catalyze the subsequent reoxidation by pyruvate. The increase in density on addition of 0.1 cc. of isocitric acid was observed until a constant value was reached, after which 0.05 cc. of pyruvic acid was added and the decrease in density measured. The reduction by isocitrate was complete in 7 minutes; the reoxidation by pyruvate was essentially complete in 1 hour and a final reading was obtained after 8 hours.

Acetaldehyde—A sample of acetaldehyde (Kahlbaum) was distilled at atmospheric pressure and a fraction collected at 20.5–21.5°. A standard solution containing about 2 mg. per cc. was prepared as described by Wagner (14). Dilute solutions were prepared just before use.

The components of the reaction mixture were essentially those described by Negelein and Wulff. 2.0 mg. of the alcohol dehydrogenase preparation and 0.092 micromole of DPNH_2 were contained in 0.05 M pyrophosphate buffer, pH 7.5, and 0.1 per cent glycine in a final volume of 1.40 cc. The reaction was essentially complete in about 8 minutes after addition of acetaldehyde.

In the presence of 0.01 M semicarbazide the reverse reaction of DPN with ethyl alcohol is 95 per cent complete and may be used for the determination of as little as 0.5 γ of alcohol.

³ Personal communication from Dr. Severo Ochoa, who kindly furnished the *dl*-isocitric acid.

TABLE I
Molecular Extinction Coefficients for DPN and TPN at 340 m μ

System	Density		Concentration of substrate <i>moles $\times$ cm.⁻¹ $\times 10^6$</i>	Molecular extinction coefficient <i>sq. cm. $\times$ mole⁻¹ $\times 10^{-3}$</i>
	Initial*	Final		
Pyruvate-DPNH ₂	0.684	0.389	47.0	6.28
"	0.683	0.440	38.5	6.31
"	0.648	0.190	73.3	6.25
Isocitrate-TPN	0.167	0.526	60.6	5.93
Pyruvate-TPNH ₂	0.494	0.212	45.0	6.27
Acetaldehyde-DPNH ₂	0.620	0.485	22.1	6.11
"	0.607	0.408	32.6	6.11
Average				6.22†

* Corrected for dilution due to substrate addition.

† This average was obtained by excluding the result with isocitrate. With the value included the average is 6.18.

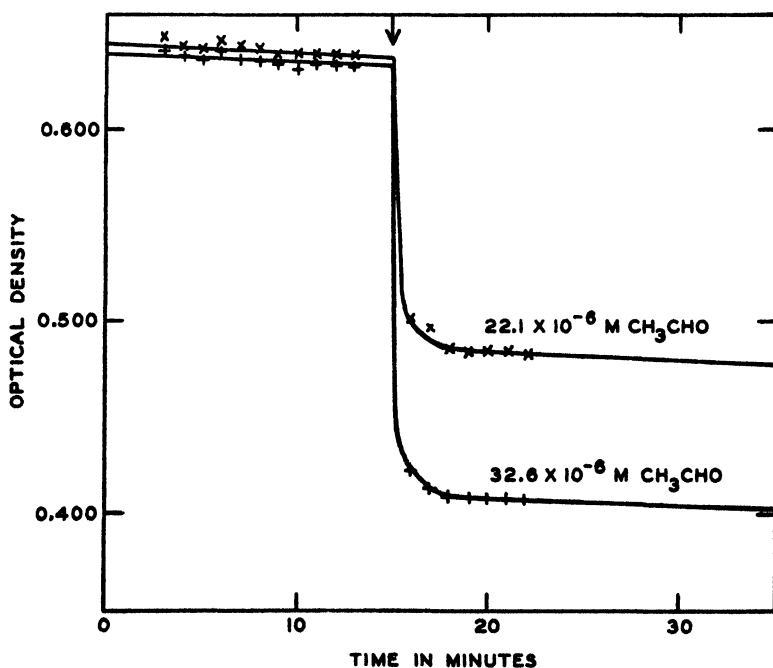


FIG. 1. The oxidation of DPNH₂ by acetaldehyde. The reaction mixture is described in the text. DPNH₂ was added to the cell at zero time. At the time indicated by the arrow, 0.04 cc. or 0.06 cc. of a 0.795×10^{-3} M acetaldehyde solution was added. The extensions of the curves beyond 23 minutes are based on points determined at 52 minutes.

TPNH₂ was not reoxidized by acetaldehyde in the presence of yeast alcohol dehydrogenase.

Results

The extinction coefficients obtained in the various reactions are shown in Table I. The values obtained agree within 2 per cent and are in excellent agreement with the coefficient reported by Ohlmeyer. In the case of isocitrate, the low value could be due to the presence of about 5 per cent of impurity in the preparation.

In the presence of high concentrations of alcohol dehydrogenase, DPNH₂ is slowly oxidized without the addition of substrate, as is shown in Fig. 1. Since this oxidation continues after the aldehyde reaction is complete, it is difficult to fix precisely the change in density due to aldehyde. The densities used in the calculations in Table I were taken at 20 minutes, shortly after the rapid phase of the reaction was completed, and the coefficients calculated must be regarded as minimum values. The extent of this error, however, is probably not greater than 1 or 2 per cent.

Using the spectrophotometric test described by Racker (15), with fructose-1,6-diphosphate as substrate, Colowick⁴ has recently measured the extinction coefficient for DPNH₂ at 340 m μ and obtained a value of 6.3×10^6 sq. cm. $\times$ mole⁻¹.

SUMMARY

1. The extinction coefficients of the pyridine nucleotides at 340 m μ have been determined from the change in light absorption on reaction with known quantities of pure substrates.

2. A molecular extinction coefficient of 6.22×10^6 sq. cm. $\times$ mole⁻¹ was obtained for the reduced forms of both diphosphopyridine nucleotide and triphosphopyridine nucleotide at 340 m μ .

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THE DETERMINATION OF RADIOACTIVE IRON IN BIOLOGICAL MATERIAL WITH PARTICULAR REFERENCE TO PURIFICATION AND SEPARATION OF IRON WITH ISOPROPYL ETHER, ASHING AND ELECTROPLATING TECHNIQUE, AND ACCURACY OF THE METHOD

By GILBERT J. VOSBURGH, LOUIS B. FLEXNER, AND DEAN B. COWIE

(From the Department of Obstetrics, The Johns Hopkins University and Hospital, the Department of Embryology, Carnegie Institution of Washington, Baltimore, and the Department of Terrestrial Magnetism, Carnegie Institution of Washington, Washington)

(Received for publication, March 4, 1948)

In the course of investigations of capillary and placental permeability to iron undertaken in this laboratory, it has been found that the several published methods (1-8) for the quantitative determination of the radioactive isotopes of iron have not met completely our requirements. Thus, the difficulties due to the low energy radiation and the low specific activity of radioactive iron have been increased by the necessity of using small amounts of radioiron if tracer concentrations are not to be exceeded, and by the necessity of dealing with certain tissues rich in calcium and phosphates. We have drawn heavily upon the experience of other investigators in the elaboration of a method which fits our needs and gives high accuracy with small amounts of radioiron. It is the plan of this paper to (1) describe in detail the procedure which is outlined below, (2) present the rationale of the method and point out certain difficulties that have been encountered, and (3) evaluate the accuracy of the method which is unique in its use of dry ashing and its use of isopropyl ether to separate iron from interfering substances.

Outline of Method

(1) Purify radioiron with isopropyl ether. (2) Determine the specific activity of radioiron. (3) Prepare biological material for assay of radioactivity. (a) Dry ash at 450-500°; (b) convert the ash to chloride salts; (c) take up material low in calcium and phosphate in ammonium oxalate solution; (d) dissolve material high in calcium and phosphate in 8 N hydrochloric acid, extract the iron with isopropyl ether, and finally remove the iron from the ether with ammonium oxalate solution; (e) electroplate the iron from ferric ammonium oxalate onto a copper disk. (4) Measure the radioactivity.

Details and Rationale of Method

Source and Purification of Radioiron—Two radioactive isotopes of iron (Fe^{55} and Fe^{59}) are available and suitable for tracer studies in biology. Although their chemical and biological characteristics are practically identical, their emitted radiations are dissimilar. For certain investigations (7) important use is made of these differences, but for our work the mixed isotopes, produced from inert metallic iron either by deuteron bombardment in the cyclotron or by neutron bombardment in the nuclear reactor at Oak Ridge, have been employed.

Radioiron prepared in the nuclear reactor has been received as ferric chloride and has been free of radioactive contaminants.

Material prepared in the cyclotron has been admixed with radioactive contaminants and purification has been necessary. Radioiron is dissolved from the target with a quantity of dilute hydrochloric acid calculated to remove only the more active superficial portion. After filtering the solution, about 1 ml. of concentrated nitric acid is added for each 0.5 gm. of iron, and the solution is then evaporated to dryness. The residue is dissolved in 20 ml. of 8 N hydrochloric acid, transferred to a separatory funnel, and shaken approximately 100 times with 20 ml. of isopropyl ether. The aqueous phase is allowed to separate completely and is then removed. The ether contains practically all of the iron, but also contains some remaining radioactive contaminants. Nine additional 20 ml. portions of 8 N hydrochloric acid saturated with isopropyl ether are successively shaken with the ether and discarded. Finally, ferric chloride, free of radioactive contaminants, is quantitatively removed from the ether by shaking it briefly with about 20 ml. of 0.1 N hydrochloric acid.

Discussion—Special care is necessary to free the iron of radioactive contaminants, particularly those with high energy radiation. Purification may be accomplished by a series of precipitations (7) with excellent results. The use of isopropyl ether, suggested to us by Thomas H. Maren, School of Medicine, The Johns Hopkins University, and based on the technique proposed by Dodson, Forney, and Swift (9), provides a method which is considerably more expeditious. Isopropyl ether possesses several advantages over ethyl ether which has long been used to separate ferric chloride from other substances. Among these are the higher extraction efficiency when small quantities of iron are present and the somewhat greater range of acid concentration permitted by isopropyl ether. We have routinely used technical grade isopropyl ether with excellent results, but it has been found that ether stored in glass containers exposed to light is not suitable for quantitative extraction. Shaking the ether with an aqueous solution of sodium sulfite will remove peroxides which may be present, but care must

be taken to remove all sulfite from the ether (10). Iron chloride must be in the higher valence state before extraction with isopropyl ether, and it is for this reason that concentrated nitric acid is added during the initial evaporation. Recovery experiments in which radioiron is used as a tracer indicate that as little as 3 mg. of iron in 25 ml. of 8 N hydrochloric acid is quantitatively extracted with two 25 ml. portions of isopropyl ether.

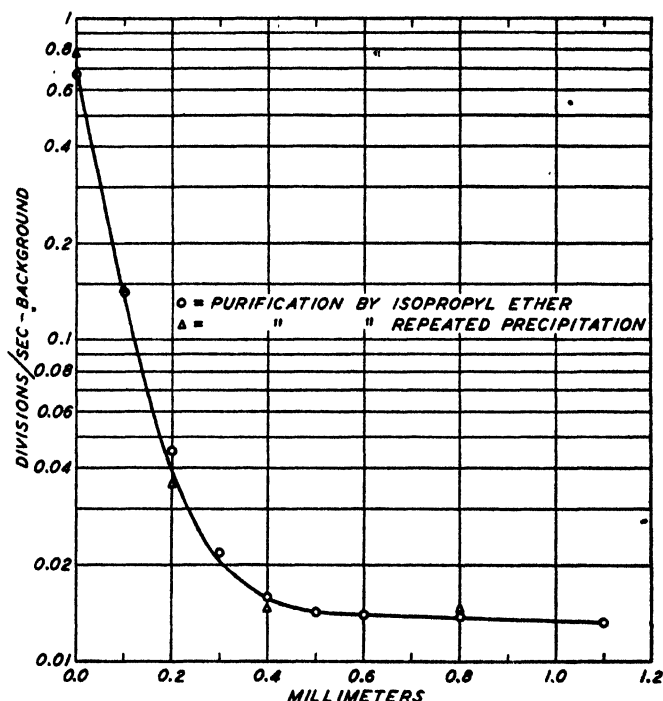


FIG. 1. A comparison of the β absorption curves of radioiron purified by repeated precipitations and with isopropyl ether. The measurements were made with an open air ionization chamber connected to a Lutz-Edelmann electrometer. The abscissa is in terms of mm. of thickness of the aluminum absorber placed between the source and the ionization chamber.

Two commonly accepted methods of ascertaining the purity of a radioactive isotope are the determination of its half life and determination of the β -particle absorption curve. The relatively long half lives (47 days and 4 years) of radioactive iron make the use of the first method somewhat tedious. We have employed the second method and find that the β -particle absorption curve of radioiron purified by extraction with isopropyl ether precisely fits the curve of iron purified by repeated precipitations (Fig. 1). In both cases the curve satisfies the known spectrum of radiation energies

of Fe^{59} (11). The absorption curves of radioactive iron prepared in the nuclear reactor have been identical both before and after purification with isopropyl ether. This finding is the basis for the statement that the substance was free of radioactive contaminants when received.

Determination of Specific Activity—The solution of ferric chloride from either the cyclotron or nuclear reactor is dried in a tared vessel, weighed, and then dissolved in an appropriate quantity of 0.1 N hydrochloric acid to give a stock solution of convenient concentration. An aliquot of this is taken for further dilution and colorimetric determination of total iron with 1,10-phenanthroline (12).

A second aliquot of the diluted stock solution is taken for electrodeposition of radioiron and determination of its specific activity, which is defined as the number of disintegrations per unit time per unit weight of material. The specific activities of the various samples of radioiron which have been used in this laboratory have ranged from 1.5 to 25 counts per second per microgram of radioiron, as measured under our standard conditions described in a subsequent section.

Treatment of Biological Material. Dry Ashing—The tissue to be analyzed is placed in a Pyrex crystallizing dish or beaker. Samples of carcass are minced with scissors to facilitate ashing. If necessary, sufficient ferric nitrate dissolved in 0.1 N nitric acid is added to raise the amount of iron in the sample to between 2 and 4 mg. 1 or 2 ml. of concentrated nitric acid are added for each 5 ml. (or gm.) of sample. With red blood cells or plasma, the acid is added before evaporating the sample to dryness in an oven at 100–110°. The carcass must be evaporated to dryness before and after addition of acid to prevent foaming. Water is first driven off in the oven, acid is then added, and the sample again dried. The vessels are now placed in a muffle furnace at room temperature and the temperature then raised to between 450–500°. After ashing overnight there is often a small amount of residual carbon. This is destroyed by moistening the ash with concentrated nitric acid, drying the residue on a hot-plate, and then returning the vessels to the hot muffle for about 30 minutes.

Discussion—Destruction of organic matter can be accomplished by one of two methods of ashing. The more commonly employed wet ashing technique is not satisfactory for our purposes; the dangers of explosion of perchloric acid are well known, and the large volume of sulfuric acid required to ash sizable samples of carcass is undesirable. Therefore, in spite of the conflicting reports about the accuracy of dry ashing, it was decided to attempt to use this method. Results are completely satisfactory when care is taken to introduce the sample into a cool muffle which is then raised to a temperature not higher than 500°. The temperature reaches 450° in 5 hours, after which there is a slow rise to 500° during the next 10 hours.

Ferric chloride is appreciably volatile even at temperatures as low as 200°, whereas iron is not lost by volatilization of ferric nitrate which is converted to ferric oxide at ashing temperature. Consequently concentrated nitric acid is added to the tissue in the oven and carrier iron is added as the nitrate in nitric acid.

Conversion of Ash to Chloride Salts—The ash is allowed to cool and is then dissolved in concentrated hydrochloric acid; 2 or 3 ml. of acid are sufficient for 5 ml. samples of red blood cells or plasma, whereas in the case of 100 gm. samples of carcass, 20 ml. or more may be required to effect solution. Free hydrochloric acid is removed by evaporation on a hot-plate or steam bath, care being taken not to heat beyond the point of dryness.

Discussion—Well ashed carcass should be chalk-white and red blood cells or plasma rust-colored after cooling. The ash is dissolved and converted to a mixture of chlorides by heating with concentrated hydrochloric acid; this process also hydrolyzes the pyrophosphates formed during ashing to soluble phosphates.

The solution is evaporated to dryness with care to avoid overheating the residue, since overheating may produce relatively insoluble basic salts and may cause loss of iron by volatilization of the chloride.

Solution of Ash Low in Phosphate and Calcium in Ammonium Oxalate—The chloride residue from 5 ml. samples of plasma or red blood cells is dissolved with a little heat and agitation in 10 ml. of saturated aqueous ammonium oxalate. The resulting solution of ferric ammonium oxalate in ammonium oxalate is transferred to the assembled electrolysis cell together with two 5 ml. washings of saturated ammonium oxalate.

Treatment of Ash High in Phosphate and Calcium—Electrodeposition of iron derived from tissues containing large quantities of calcium and phosphate requires additional preparation of material because, in the alkaline medium produced during electrolysis, basic iron phosphates are precipitated and iron thereby lost. This difficulty is met by separation of iron with isopropyl ether from interfering substances. The chloride residue from the carcass is dissolved in 8 N hydrochloric acid. Approximately 1 ml. of acid will be required for each gm. of tissue originally taken. Careful heating to effect solution is permissible, but the concentration of the acid should not be significantly changed. The solution is transferred to a separatory funnel and the vessel washed with two 10 ml. portions of isopropyl ether which are then added to the separatory funnel for extraction of the ferric chloride. After shaking about 100 times the aqueous phase is allowed to separate and is transferred to a second separatory funnel, where the extraction is repeated with an additional 20 ml. of isopropyl ether. The ether fractions are combined and the iron extracted from the ether with a single 20 ml. portion of saturated aqueous ammonium oxalate. Any considerable precip-

itate of calcium oxalate is removed when the solution of ferric ammonium oxalate is filtered directly into the electrolysis cell.

Electrodeposition of Iron—The anode is introduced into the electrolyte, centered over the cathode, and adjusted to an arbitrarily chosen but reproducible vertical position. A potential of 8 volts is applied and each cell initially draws approximately 200 milliamperes, which is a current density of about 6 amperes per 100 sq. cm. After 3 hours of plating, the anode and the side walls of the cell are washed down with a few ml. of ammonium oxalate solution, and the current continued for another half hour. A 1 ml. sample of the solution is withdrawn to be tested for residual iron. It is acidified with 4 drops of concentrated sulfuric acid and then boiled for a moment to make certain there is no suspended iron hydroxide in the solution. 2 drops of a 0.1 per cent aqueous 1,10-phenanthroline solution and a drop of 10 per cent aqueous solution of hydroxylamine hydrochloride are added. The pH is adjusted to between 6 and 9 with concentrated ammonium hydroxide. Color development is immediate and as little as 0.05 γ of iron per ml. of solution can be detected. After a total of 3.5 hours electrolysis, the qualitative test for iron is usually negative or only faintly positive and electrolysis is discontinued. The cells are rinsed with distilled water and alcohol, after which the copper disks are removed and air-dried. With reasonable care the iron is plated in a smooth, densely adherent film, silvery gray in color.

Discussion—Electroanalysis of iron from aqueous solutions of the salts of the inorganic acids is unsatisfactory because, in the rather strongly acid medium necessary to prevent precipitation of the hydroxide, hydrogen is much more readily displaced from solution than iron. However, electrodeposition is eminently satisfactory from solutions of the ammonium double salts of certain organic acids; in these the hydrogen ion concentration can be kept low, and the ionization constant of the complex salt is such that the solubility product of iron hydroxide is not exceeded. The organic acids commonly used are citric, tartaric, and oxalic, and the last is reputed to give the best results (13). In practice the iron chloride or sulfate is dissolved in a solution of ammonium oxalate to produce ferric or ferrous ammonium oxalate. Although most methods recommend that the complex salt be formed from ferrous or ferric sulfate, we have found the use of the chloride to be simpler. The sulfate salts of some of the tissue constituents are relatively insoluble in ammonium oxalate; moreover, electrodeposition from the sulfate is sometimes unsatisfactory due, probably, to the difficulty of complete removal of sulfuric acid.

During electrolysis the pH of the solution rises from the initial value of about 4.5 to 8 or 9. No attempt is made to regulate the pH by the addition of oxalic, hydrochloric, or sulfuric acid, and no difficulty is encountered from

the precipitation of iron hydroxide when as much as 14 mg. of iron is plated from a solution whose total volume is 20 ml.

The length of time for complete electrolysis can be substantially shortened by using current densities as high as 25 or 35 amperes per 100 sq. cm. (3, 7). Under these conditions, however, the solution must be stirred to obtain satisfactory deposition of metallic iron. Theoretically the time can be diminished if the iron is in the ferrous state. For the sake of simplicity and because it has not been inconvenient to electroplate for a period of 3.5 hours, we have not stirred the solution nor reduced the iron.

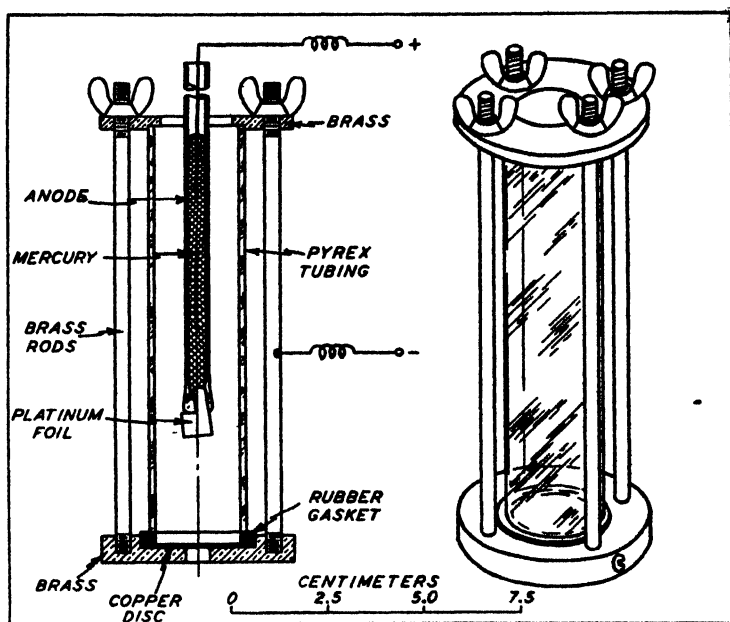


FIG. 2. The assembled electrolysis cell and cross-sectional drawing to show components. The hole in the bottom plate is to aid in the removal of the copper disk.

The electrolysis cells, a drawing of which appears in Fig. 2, were designed to assure precise reduplication of the distribution of the electroplated iron from cell to cell and to make unnecessary the use of an insulating paint (3). The latter, in our hands, has not consistently protected against deposition of iron on the sides of the disks, with consequent loss of measurable radioactivity. The cells are not difficult to construct and the drawing is almost self-explanatory. It should be mentioned that the rods which hold the top and bottom brass plates are set eccentrically, so that the glass tubing, rubber gaskets, and copper disks can be removed after simply loosening the

wing nuts at the top. Since the disk and gasket fit into the same shallow depression, they are automatically centered with respect to one another and the iron is always distributed over the same area of the disk. The use of a rubber gasket makes unnecessary not only a ground glass junction between the disk and the glass side walls but also the use of sealing compounds to prevent leakage of electrolyte (5). The copper disks, which serve not only as the mechanical bottom of the cell but also as the cathode, are made from annealed sheet copper 0.032 inch thick and are 30 mm. in diameter. A punch and die may be used or the disks may be turned out on a lathe. Since the rubber gasket covers a portion of the surface, the effective plating diameter over which the iron is distributed is 21 mm. It is important that the surface be nearly plane, since irregularities will produce errors in the counting rate. Steel wool, which we have found more convenient than nitric acid, is used to clean the surface of the disks.

Great care must be exercised to protect against contamination of the cells. After each electrolysis the glass tubing and rubber gaskets are allowed to soak overnight in dilute hydrochloric acid, then washed with soap and water, and rinsed with distilled water. The anodes are immersed after an electrolysis in test-tubes containing a solution of inert ferric chloride in dilute hydrochloric acid. The solution is rinsed off with distilled water just prior to use.

A selenium rectifier coupled to a heavy duty filament transformer has been used as a source of direct current. A rheostat is incorporated in the circuit to regulate the voltage applied to the cells. Customarily a bank of eight cells is in use, but, if fewer than this number are in operation, the rheostat is of sufficient resistance and power capacity to compensate for the decreased current drawn.

The current carried by each cell will be determined by several factors, among which are the composition and temperature of the electrolyte, the distance between the anode and cathode, and the applied potential. Thus, initially 200 milliamperes flow through each cell, but soon the temperature of the electrolyte rises and the current increases to about 280 milliamperes.

The spatial relationship of the anode and cathode influences not only the current drawn by the cells but also the distribution of the electroplated iron on the cathode. The anode should be approximately centered over the cathode and the anode-cathode distance must be kept nearly constant from cell to cell and in successive electrolyses. With the separation of the electrodes reduced to 0.5 cm., we find that the counting rate is 18 per cent greater than when the same amount of radioiron is electroplated in cells with an anode-cathode distance of 3 cm. This effect is explained by the deposition of a larger proportion of radioiron on the central portion of the disk with smaller anode-cathode distances and the more efficient counting

of the Geiger-Müller tube placed over a smaller source. The uniform density of the radioautograph reproduced in Fig. 3 clearly demonstrates the even distribution of radioiron on the copper disk.

Either 1,10-phenanthroline or 2,2'-bipyridyl is used to determine the end-point of electrolysis. It should be recalled that the thiocyanate test for iron is relatively insensitive in the presence of oxalate; in our hands the ammonium sulfide test has not been as satisfactory as *o*-phenanthroline or bipyridyl. We have found that after 3.5 hours of electrolysis approximately 1.3 γ of iron remain in 20 ml. of plating solution, or about 0.04 per cent of the total of 3 mg. originally present.

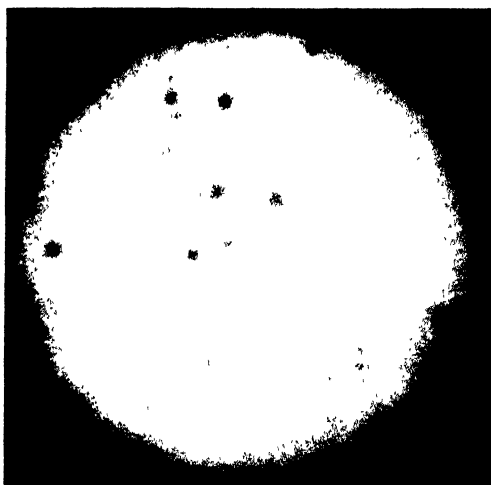


FIG. 3. Representative radioautograph of electroplated radioiron

Uniformity of performance among our eight electrolysis cells was tested by measuring the radioactivity of a constant amount of radioiron electroplated in each of the cells. Approximately 42,025 counts were taken from each disk to give a standard deviation, due to fluctuation of radioactive disintegration alone, of 205. Six of the eight determinations were within 1 standard deviation from the mean, while the seventh and eighth were within 2 and 2.7 standard deviations respectively. Since the observed variation was no greater than that expected from fluctuation of radioactive disintegration, we conclude that the eight electrolysis cells give reproducible results.

Measurement of Radioactivity—The bell-shaped, self-quenching Geiger-Müller tube is filled with argon and an alcohol vapor and has a mica window 2.9 cm. in diameter weighing 3.3 mg. per sq. cm. Such a tube is insensitive to the characteristic radiation produced in the disintegration of Fe^{55} and

counts only the radiation of Fe^{59} . The copper disk, on which the radioiron is plated, is rigidly supported immediately beneath the counting tube window. The counting rate of the unknown is determined and the background rate subtracted. The amount of radioiron present on the disk is easily determined by referring the counting rate, less the background, of the sample to the counting rate of a known quantity of the radioiron having the same specific activity. The total number of disintegrations counted in any particular determination will depend upon the precision desired, as described in the discussion of "Accuracy of method."

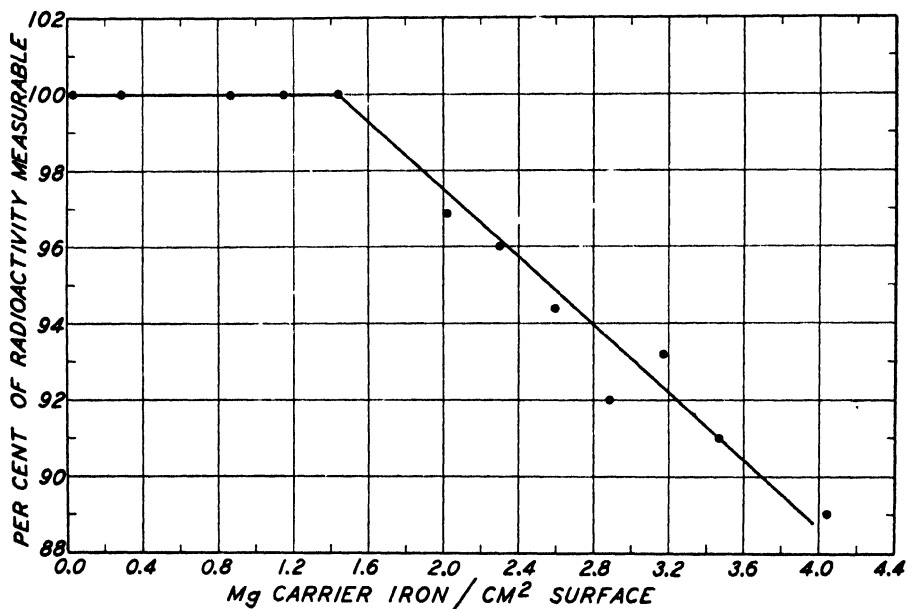


FIG. 4. Self-absorption of radiation from fixed amount of radioiron by increasing amounts of carrier iron.

Discussion—Since samples of biological material may contain widely varying amounts of intrinsic iron, it is imperative that one be in position to correct the counting rate for self-absorption of radiation. As is apparent from Fig. 4, we find no significant self-absorption when quantities of inert iron less than 1.4 mg. per sq. cm. are coplated with negligible weights of radioiron. When more than this amount is present, the counting rate decreases linearly, and 4 mg. of carrier iron per sq. cm. give a counting rate 11 per cent lower than the maximum. Although the value for self-absorption of the β radiation depends largely upon the thickness of the coplated inert iron, it will vary with other factors, such as the characteristics of the

individual Geiger-Müller tube, the distance between the source of radiation and the tube, and the distribution of the isotope with respect to the counter window. Thus, it becomes apparent that the absorption curve determined in one laboratory is not precisely applicable to data obtained in another.

Accuracy of Method

Recovery of a known quantity of radioiron was used to test each step of the procedure, and over-all accuracy was tested by recovery of radioiron from plasma, red blood cells, and fetal carcass carried through the entire

TABLE I
Recovery of Radioactive Iron

Average recovery in the thirteen experiments is 18.33 γ or 100.17 per cent with a standard deviation of 0.09 γ . The standard deviation, due to fluctuation in radioactive disintegration alone, is 0.7 per cent, which is equivalent to 0.13 γ when related to our standards as explained in the text.

Sample No.	Sample	Total counts		Time		Counts per sec.		Per cent recovery
		Sample	Stand- ard	Sample	Stand- ard	Sample	Stand- ard	
				min.	min.			
1	Plasma 1.0 ml.	43,904	43,880	24	24	30.489	30.472	100.05
2	" 1.0 "	44,342	43,880	24	24	30.793	30.472	101.05
3	" 1.0 "	43,904	43,880	24	24	30.489	30.472	100.05
4	" 1.0 "	38,288	38,353	25	25	25.525	25.569	99.83
5	" 1.0 "	37,952	37,686	25	25	25.301	25.124	100.70
6	" 1.0 "	38,457	38,688	25	25	25.638	25.792	99.40
7	" 1.0 "	38,187	38,149	25	25	25.458	25.433	100.10
8	Red blood cells 3.0 ml.	42,124	49,158	24	28	29.253	29.261	99.97
9	" " " 3.0 "	44,224	29,246	30	20	24.569	24.372	100.81
10	" " " 2.5 "	40,749	40,504	26	26	26.121	25.964	100.60
11	Carcass 25 gm.	42,227	42,436	24	24	29.324	29.469	99.51
12	" 25 "	43,253	43,305	24	24	30.037	30.073	99.88
13	" 50 "	43,987	43,867	25	25	29.325	29.245	100.27

method. It has been shown in the discussion of "Electrodeposition of iron" that electroanalysis of iron is essentially complete, and that reproducibility is such that variation from sample to sample may be accounted for by fluctuation in radioactive disintegration. The same accuracy is obtained when known quantities of radioiron are extracted from 8 N hydrochloric acid with isopropyl ether, taken up in saturated aqueous ammonium oxalate, and then electroplated. The data from recovery experiments in which 18.3 γ of radioiron were added to samples of biological material are listed in Table I. The over-all accuracy obtained when electrolysis, separation with isopropyl ether, and ashing are combined is so completely satis-

factory that variations among samples are no greater than can be explained by the variation in radioactive disintegration. Similar results have been obtained at the National Institute of Health by Endicott and his coworkers who have successfully used the method described in this paper after subjecting it to careful test.¹

Discussion—Interpretation of differences in the counting rate among samples was important to us in testing the reproducibility of electroanalysis and in evaluating data obtained from recovery experiments. Were the observed differences in counting rate from sample to sample of such magnitude as to be explained by the fluctuations inherent in radioactive disintegration? Or were they due to real differences in the amount of radioiron, resulting from deficiencies of the method? Since radioactive disintegration is a random process and fits Poisson's law, the standard deviation, due to fluctuation in radioactive disintegration alone, is given by the square root of the number of disintegrations counted. This also may be taken as a sufficiently accurate estimation of the standard deviation of the counts from a strong sample. Thus, if 10,000 disintegrations from such a sample are counted, the standard deviation is 100, or 1 per cent of the counts; if 2500 counts are taken, the standard deviation, although numerically smaller, rises to 2 per cent of the count. The results of measurements are almost invariably expressed as rates and the standard deviation of the counting rate from a strong sample is given by $\sqrt{n/t^2}$, where t is the time interval over which the total count, n , was obtained. When weak samples are measured, it is necessary to consider fluctuation of the background counting rate as well as that of the sample. If we have a count n_s in time t_s from a weak sample (including the background), and a count n_b in time t_b from the background, then the counting rate of the sample alone is estimated as $(n_s/t_s) - (n_b/t_b)$ and the standard deviation is approximated by $\sqrt{(n_s/t_s^2) + (n_b/t_b^2)}$.

The data of Table I enable testing the over-all accuracy of the method, as represented by the per cent recovery, and comparison of this with the inherent uncertainty arising from counting variations. The mean recovery in the thirteen experiments is 100.17 per cent, and the standard deviation from the mean is 0.5 per cent. This may be compared with the standard deviation arising from counting variation alone. Recovery is calculated as

$$r = \frac{n_u}{t_u} \div \frac{n_s}{t_s} = \frac{n_u \times t_s}{t_u \times n_s}$$

where t_u and n_u refer to the time interval over which the count of the sample was measured and t_s and n_s are the corresponding values of the standard to

¹ Endicott, K. M., personal communication.

which the sample is referred. The standard deviation of this ratio (since n_u and n_s are independent and are both large) is approximated by

$$\sqrt{\frac{n_u}{i_u^2} + \frac{n_s}{i_s^2}}$$

In the recovery experiments n_u and n_s are approximately equal and of the order of 40,000, so that $\sigma_r = 0.007$ or 0.7 per cent. It is now apparent that the actual variation of the per cent recovery (0.5 per cent) is slightly less than is anticipated from counting variation alone. This does not imply, of course, that the chemical treatments and manipulations necessary to prepare the biological samples for measurement are without variation, but merely that the variations so introduced are small compared with those inherent in counting.

In general, investigation of variability arising from sources other than fluctuation in radioactive disintegration requires a comparison of the observed variability with that expected from counting variations, and two methods have now been described. Thus, in the discussion of "Electrodeposition of iron" the reproducibility of the eight cells was described by comparison of the observed distribution of counts about their mean with the theoretical distribution, and in the foregoing paragraph the standard deviation in the recovery experiments was compared with the anticipated variation due to fluctuation in radioactive decay. A more convenient test is to compute χ^2 , which is given by $\Sigma (x-m)^2/m$, where x is the numerical value of a single count and m is the arithmetic mean of the series. The quantity χ^2 has a known probability distribution depending only on the number of observations in the series (14). If, then, the value of χ^2 is so large that it is not likely to have arisen by chance, it must be concluded that other sources of variation are significantly greater than that due to fluctuation in radioactive disintegration. Application of the χ^2 test to the data obtained in the case of the eight electrolysis cells and the recovery experiments permits the conclusion that there is no reason to suspect that the method described for the determination of radioiron does not give accurate results.

SUMMARY

A method for the determination of radioactive iron in biological material including carcass is described. Dry ashing is substituted for the more commonly employed wet ashing technique, and isopropyl ether is used to purify radioiron and to separate iron from substances that interfere with electrodeposition.

The recovery of known amounts of radioiron added to samples of red blood cells, plasma, and fetal carcass is complete. The precision of the

method is such that the values for recovery lie within the limits imposed by statistical fluctuation in radioactive disintegration.

We are indebted to Dr. Rowland V. Rider and Dr. Charles P. Winsor of the School of Hygiene and Public Health, The Johns Hopkins University, for their assistance with the section of the paper describing the statistical variations.

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AVAILABILITY OF S-BIS(γ -AMINO- γ -CARBOXYPROPYL)-SULFIDE (HOMOLANTHIONINE) IN SULFUR METABOLISM OF THE RAT*

BY JAKOB A. STEKOL AND KATHRYN WEISS

(From the Lankenau Hospital Research Institute and The Institute for Cancer Research, Philadelphia)

(Received for publication, April 10, 1948)

L-Cystathionine has been shown to be available to the rat in lieu of L-cystine, while L-allo-cystathionine was similarly available in lieu of methionine in the presence of dietary choline (1). Studies *in vitro* revealed the formation of cysteine and homocysteine from L-cystathionine and L-allo-cystathionine respectively in the presence of rat liver slices (2). Other experimental findings place cystathionine in the position of a physiological intermediate in the transformation of methionine to cysteine in the rat (3) and of cysteine to methionine in *Neurospora* (4). Thus, originally an artifact prepared by Küster and Irion (5) by treatment of wool with Na_2S , cystathionine assumed a strategic position in sulfur metabolism.

Lanthionine, another artifact prepared by Jones *et al.* (6) by treatment of keratins with Na_2CO_3 , is of physiological interest, since recent studies have shown that apparently the L isomer of DL-lanthionine stimulated the growth of rats on low casein diets (7), although the meso form of lanthionine was not available for the purpose (8). These data suggested the cleavage of L-lanthionine *in vivo*, and raised the question of the position of L-lanthionine in sulfur metabolism.

We reported recently the synthesis of mixed isomers of cystathionine and of the next higher homologue of lanthionine, S-bis(γ -amino- γ -carboxypropyl)sulfide, which we named homolanthionine (9). Structural similarity of homolanthionine to cystathionine and to lanthionine suggested possible cleavage of the thio ether amino acid to homocysteine and, perhaps, to homoserine *in vivo*. Although homolanthionine is another artifact, in view of the biochemical history of cystathionine and of lanthionine it appeared to be of interest to investigate the availability of this compound to the rat for growth purposes. Such a study is the object of this report.

EXPERIMENTAL

The low casein diet employed in the first series of experiments had the following percentage composition: Labco vitamin-free casein 8, corn-starch

* Aided by a grant from the Pardee Foundation.

47.1, sucrose 15, inorganic salts (10) 4, Crisco 20, and cod liver oil 5. The B complex vitamins and the liver extract were added to this diet (referred to in Table II as Diet C-8) in the amounts indicated in Table I.

In the second series of experiments a diet was employed in which the sole source of protein nitrogen was supplied by a mixture of amino acids, the composition of which is shown in Table I. The amino acid mixture, with some modifications, was essentially that of Rose and Womack (11). In

TABLE I
Amino Acid Mixture

The vitamins and the amino acids were thoroughly ground together before the addition of the other ingredients of the diet indicated in the text.

	gm.		gm.
Glycine	0.1	Hydroxyproline	0.1
Alanine*	0.4	Tryptophan	0.4
Serine*	0.4	Aspartic acid	0.2
Valine*	2.0	Glutamic "	2.0
Leucine*	2.6	Lysine·HCl	1.9
Isoleucine*	1.8	Arginine·HCl	0.6
Threonine*]	1.4	Histidine·HCl	0.7
Phenylalanine*	1.0	NaHCO ₃	1.3
Proline	0.2		
Total			17.1

Vitamins per 100 Gm. of Complete Diet

	mg.		mg.
Thiamine·HCl	1	Ca pantothenate	5
Riboflavin	2	p-Aminobenzoic acid	100
Pyridoxine·HCl	1	Inositol	100
Folic acid	1	Lilly liver extract	700
Nicotinamide	1		

* Racemic amino acids.

addition to the vitamins and the liver extract listed in Table I, the diet consisted of the following ingredients: amino acid mixture 17.1, dextrin 30.0, sucrose 15, inorganic salts (10) 4, agar 2.0, Crisco 26.0, and cod liver oil 5.0 gm. per 100 gm. of the diet.

The supplements were mixed with the experimental diet in the following amounts (per cent): choline hydrochloride 0.2, L-cystine 0.4, DL-methionine 0.2, inactive homocystine 0.44, inactive homolanthionine 1.0. The diets and water were fed *ad libitum* and a record of food consumption was kept. 25 to 30 day-old albino male rats of the Wistar strain born and raised in

this laboratory were used. The animals were housed in individual metabolism cages and weighed twice a week.

All the amino acids used were of established analytic purity. Homolanthionine was prepared by the recently described method (9). Neither the thiol nor the disulfide group could be detected in the preparation by the sodium-cyanide-nitroprusside test. We wish to express our appreciation to Dr. H. R. Snyder of the University of Illinois for the generous gift of 3,6-bis(β -chloroethyl)-2,5-diketopiperazine, which enabled us to prepare a substantial quantity of homolanthionine for feeding experiments.

TABLE II
Availability of Homolanthionine to Rats for Growth on Low Casein Diet

Group No.*	Initial weight	Final weight	Gain per day	Days on diet	Food intake per day	Supplements to Diet C-8
	gm.	gm.	gm.		gm.	per cent
1	56	158	1.9	54	9.3	1.0 homolanthionine
	158	165	1.0	7	9.1	None
2	62	132	1.6	43	8.1	0.2 choline + 1.0 homolanthionine
	132	138	0.7	9	9.1	0.2 "
3	63	137	1.1	64	8.8	0.2 "
	137	163	1.9	14	9.0	0.2 " + 1.0 homolanthionine
	163	166	0.2	14	8.7	0.2 "
4	87	154	3.2	23	10.2	0.44 homocystine
	154	149	-0.6	8	8.7	None
5	91	139	3.2	15	9.7	0.2 choline + 0.44 homocystine
	139	150	0.5	19	6.2	0.2 "
6	59	204	2.6	55	9.6	0.4 cystine
7	76	190	2.6	44	8.5	0.5 methionine
8	54	139	1.1	75	8.0	None
9	50	126	1.1	72	8.4	0.2 choline

* Seven litters of rats were used. Groups 1, 2, and 3 each consisted of six rats, all others of three animals each. The animals were kept in individual metabolism cages. The data are average values for each group.

Results

As is apparent from the data presented in Table II, homolanthionine stimulated the growth of rats when it was incorporated into the low casein diet. This stimulation of growth by homolanthionine was somewhat less pronounced than that induced by the administration of equivalent amounts of homocystine, L-cystine, or DL-methionine (sulfur equivalents). Withdrawal of homolanthionine from the diet was followed by immediate retardation of growth. Addition of choline to the diet did not significantly increase the rate of growth of rats which ingested homolanthionine.

Since the low casein diet was ample in methionine and low in cystine, the stimulation of growth by homolanthionine could have been a result of the transformation of homolanthionine to either cystine or methionine, or both. We therefore carried out additional studies on homolanthionine with diets which were free either of cystine or methionine, in order to evaluate the

TABLE III

Availability of Homolanthionine to Male Rats for Growth on Diets Free of Cystine But Adequate in Methionine

Rat No.	Initial weight	Final weight	Gain per day	Days on diet	Food intake per day	Supplements added to amino acid mixture diet
	gm.	gm.	gm.		gm.	per cent
64	53	41	-1.7	7	3.6	0.2 choline
	41	70	0.98	30	5.4	0.2 " + 0.2 methionine + 1.0 homolanthionine
	70	74	0.3	14	5.0	0.2 choline + 0.2 methionine
	74	84	1.25	8	7.0	0.2 " + 0.2 " + 1.0 homolanthionine
65	48	40	-1.1	7	2.3	0.2 choline
	40	45	0.62	8	4.0	0.2 " + 0.2 methionine
	45	60	0.90	17	4.5	0.2 " + 0.2 " + 1.0 homolanthionine
	60	60	0.0	12	3.8	0.2 choline + 0.2 methionine
	60	79	1.1	16	5.4	0.2 " + 0.2 " + 1.0 homolanthionine
66	45	38	-1.0	7	2.6	0.2 choline
	38	63	0.4	56	4.0	0.2 " + 0.2 methionine
58	73	52	-1.3	16	3.0	None
	52	81	1.26	23	4.4	0.2 methionine + 1.0 homolanthionine
	81	88	0.3	24	5.4	0.2 methionine
59	51	36	-1.0	16	2.7	None
	36	57	0.91	23	3.9	0.2 methionine + 1.0 homolanthionine
	57	60	0.13	24	4.0	0.2 methionine

The data on each rat are representative of those obtained on three animals.

availability of homolanthionine for growth in the absence of either of the sulfur amino acids in the diet.

Table III illustrates the data obtained on feeding homolanthionine to rats which were maintained on a diet which contained 0.2 per cent of methionine, an amount of methionine which, although insufficient to produce growth, was adequate to permit growth when sufficient cystine was added. In every case the addition of homolanthionine to this diet was followed by stimulation of growth, although the rate of growth was not optimal. Addi-

tion of choline to the homolanthionine diet did not further increase the growth rate.

Table IV summarizes the data obtained on feeding homolanthionine to rats which were maintained on a diet which contained 0.4 per cent cystine and no methionine. In the absence of choline in the diet, homolanthionine

TABLE IV
*Availability of Homolanthionine to Male Rats for Growth on Diets
Containing Cystine But Free of Methionine*

Rat No.	Initial weight	Final weight	Gain per day	Days on diet	Food intake per day	Supplements added to amino acid mixture diet
	gm.	gm.	gm.		gm.	per cent
56	58	50	-4.0	2	2.0	None
	50	45	-0.36	14	3.6	0.4 cystine + 1.0 homolanthionine
	45	60	0.94	16	4.3	0.2 choline + 0.2 methionine + 1.0 homolanthionine
	60	50	-1.0	10	2.6	0.2 choline
	50	54	0.2	21	2.8	0.2 " + 0.2 methionine
57	61	51	-5.0	2	2.0	None
	51	40	-0.58	19	2.7	0.4 cystine + 1.0 homolanthionine
	40	45	0.30	18	3.2	0.2 choline + 0.4 cystine + 1.0 homolanthionine
	45	49	0.16	24	4.0	0.2 choline + 0.2 methionine
54	58	47	-5.5	2	2.0	None
	47	76	1.5	19*	4.8	0.4 cystine + 0.44 homocystine
60	66	57	-4.5	2	2.8	0.2 choline
	57	105	3.4	14	6.8	0.2 " + 0.4 cystine + 0.44 homocystine
	105	75	-1.58	19	3.6	0.2 choline
	75	77	0.12	16	3.0	0.2 " + 0.4 cystine + 1.0 homolanthionine
	77	68	-0.75	12	6.0	0.2 choline + 0.4 cystine
62	61	48	-6.5	2	1.5	0.2 "
	48	63	0.4	40	3.1	0.2 " + 0.4 cystine + 1.0 homolanthionine
	63	56	-0.63	11	4.3	0.2 choline + 0.4 cystine

* All rats died between the 12th and 23rd days. The data for each rat are representative of those obtained on three animals.

did not prevent rapid loss in weight of all animals. The addition of choline and homolanthionine to the diet checked the loss in weight and stimulated a slow gain in weight comparable to that produced by feeding 0.2 per cent methionine alone. The stimulation of growth by homolanthionine, fed together with choline and cystine, was definitely less pronounced than that obtained on feeding homolanthionine together with minimal amounts

of methionine (0.2 per cent). All rats rapidly lost weight when homolanthionine was withdrawn from the diet which furnished 0.4 per cent cystine and 0.2 per cent choline.

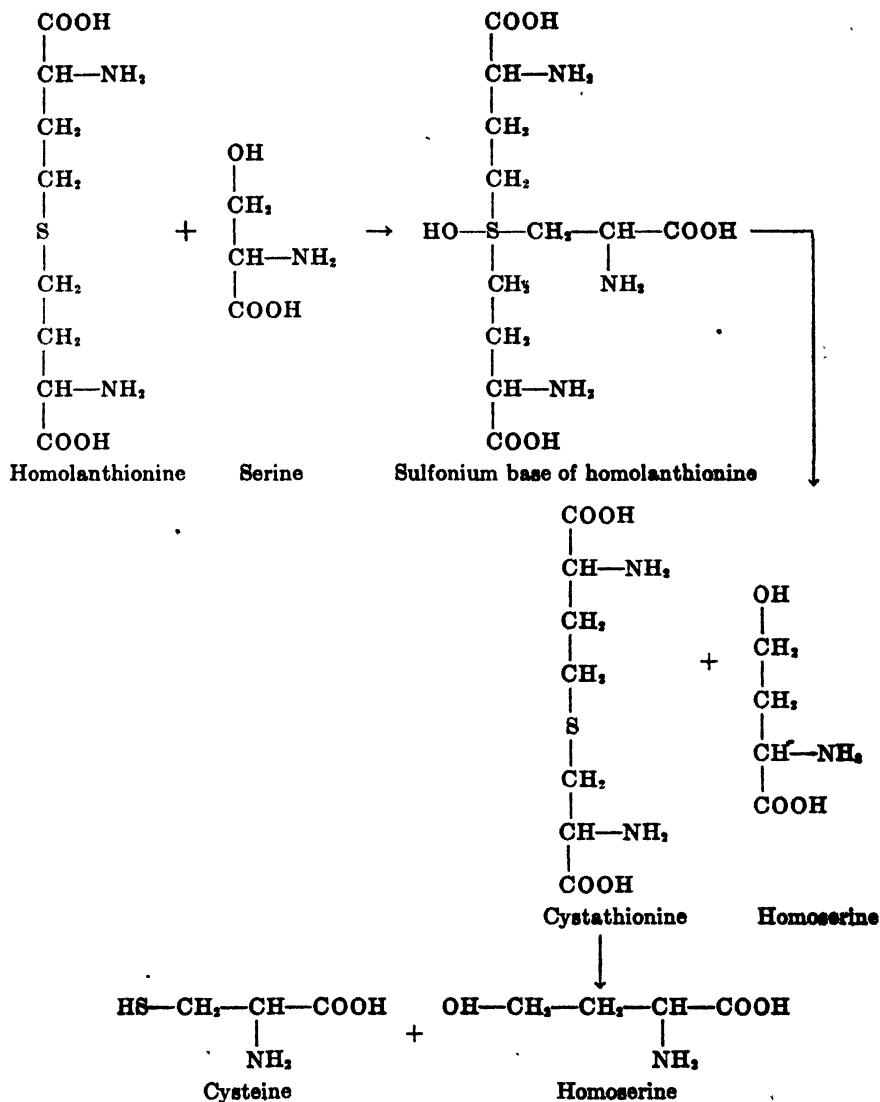
DISCUSSION

Since our preparation of homolanthionine consisted of a mixture of DL- and meso forms (9), no appraisal of the activity of the individual diastereoisomers of homolanthionine can be made on the basis of the results presented here. The results warrant the conclusion, however, that our preparation of homolanthionine contains one or more physiologically active forms which are available to the rat in sulfur metabolism. The evaluation of the activity of each of the isomeric forms of homolanthionine *in vivo* and a study of the mechanism of cleavage of homolanthionine by tissue slices would, perhaps, elucidate the chemistry which is involved in the physiological availability of homolanthionine to the rat. This is at present in progress.

Whether homolanthionine is a normal physiological product (in view of its availability to the rat) is hypothetical. It is within the realm of possibility, however, that 2 molecules of methionine could give rise *in vivo* to homolanthionine with the formation of methyl mercaptan, or dimethyl sulfide and hydrogen sulfide. The latter products have been found in animal tissues and excreta.

The data presented here cannot be easily explained on the basis of the assumption that homolanthionine (as used by us) is cleaved *in vivo* to yield homocysteine. Very poor growth was obtained with homolanthionine on diets which furnished choline and cystine, whereas with homocysteine instead of homolanthionine good growth was obtained under similar conditions. Yet, homolanthionine stimulated the growth on diets containing minimal amounts of methionine. It is possible that one of the isomers of homolanthionine yields homocysteine *in vivo*, which in the presence of choline is converted to methionine, and thereby increases the amount of methionine available to the rat ingesting a diet containing minimal amounts of methionine. Such a formation of homocysteine from one of the isomers of homolanthionine would have to be small, indeed, since in the presence of choline and cystine, but no methionine in the diet, this amount of formed methionine is insufficient to stimulate the growth, although maintenance of weight is secured.

It would appear that an alternate explanation of the activity of at least one isomer of homolanthionine present in the mixture must be made. And this could be based on the assumption of cysteine formation from homolanthionine without the preliminary cleavage of the thio ether to homocysteine. A working hypothesis is proposed in the accompanying graphic form.



According to this scheme it is assumed that homolanthionine and serine would give a hypothetical sulfonium base of homolanthionine, which is analogous to that proposed by Toennies (12) for methionine and serine. The sulfonium base of homolanthionine would then cleave to homoserine and cystathionine. The latter would then yield cysteine and another molecule of homoserine. This scheme would tentatively explain the activity of our preparation of homolanthionine, in that it suggests the forma-

tion of cystathionine and cysteine from homolanthionine without the preliminary formation of homocysteine. Obviously, this proposed scheme does not in any way conflict with the present status of the problem of conversion of methionine to cysteine *in vivo*. It does, on the other hand, point to another possible path of metabolism of methionine, namely via homolanthionine, as we have already stated. Further work is clearly necessary along the lines suggested by this hypothesis, particularly with regard to the proposed formation of a sulfonium base of homolanthionine and, perhaps, of methionine proposed by Toennies (12). Plans along these lines are now being carried out.

SUMMARY

A mixture of DL- and meso forms of S-bis(γ -amino- γ -carboxypropyl)sulfide (homolanthionine) stimulated the growth of rats which were maintained either on a low casein diet or an amino acid mixture diet which contained only minimal amounts of methionine and no cystine. Poor growth was obtained when homolanthionine was fed on an amino acid mixture diet which contained cystine and choline, but no methionine. The significance of these results is discussed, and a working hypothesis is presented in which homolanthionine can be expected to give rise to cysteine, via cystathionine, without undergoing a preliminary cleavage to homocysteine *in vivo*.

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MECHANISM OF THE FERMENTATION OF LACTOSE BY YEASTS

By MORRISON ROGOSA*

(From the Bureau of Dairy Industry, Agricultural Research Administration, United States Department of Agriculture, Washington)

(Received for publication, April 17, 1948)

There are two fundamental theories concerning the fermentation of disaccharides. One presupposes an "indirect" and the other a "direct" fermentation. A theory proposed by Fischer (1-3) was that no compound sugar is fermented directly as such, but that the compound sugar is cleaved by appropriate hydrolytic enzymes into its monose constituents, the latter actually being fermented as such. Briefly stated this is the classical theory of the indirect fermentation of compound sugars.

Leibowitz and Hestrin (4) stated that:

"Fischer's explanation, owing largely perhaps to the high authority of its author, was widely accepted. Essentially a speculative theory based on an attractive correlation, it assumed in the minds of both chemists and biologists the status of a proved fact. Even though Fischer himself had not even attempted to subject his theory to crucial quantitative test, the theory went almost unchallenged for nearly a generation. Moreover, its scope was broadened and the belief became general that the Fischer mechanism described not only alcoholic fermentation of oligosaccharides by yeast, but also the fermentations of oligosaccharides by bacteria, and equally the glycolysis of compound sugars by tissues of the higher forms of life. Laborde's [(5)] early criticism of the Fischer view in its application to sucrose metabolism in heterofermentative lactic acid bacteria was simply ignored."

Willstätter and Oppenheimer (6) observed that lactose-fermenting yeasts may ferment lactose at a faster rate than they ferment the monose constituents of lactose, glucose, and galactose, either individually or in mixtures in concentrations equivalent to those of lactose. They found that the concentration of lactase in yeast extracts was insufficient to hydrolyze lactose at a rate equivalent to the fermentation of lactose by the intact cells. In some instances the lactase content of yeast extracts was negligible or zero. Because technical difficulties make it impossible to conduct experiments on lactase with the intact cell, certain objections can be raised to a positive statement that direct fermentation occurs. Thus, the evidence for direct fermentation presented by Willstätter is suggestive and inferential, but not conclusive.

Hestrin (7) demonstrated that lactose can be fermented more rapidly than hexoses by using a yeast strain from Palestine *Leben*, and Myrbäck

* Present address, National Institute of Health, Bethesda, Maryland.

and Vasseur (8) obtained a similar result with *Saccharomyces fragilis* and *Torula cremoris*.

Kluyver and Custers (9) noted that *Blastodendron intermedium* fermented lactose, but did not respire in it, whereas the organism both fermented and respired in glucose. The lack of respiration in lactose would indicate that glucose, on which the cells do respire, was not formed.

Wright (10, 11) has suggested a direct fermentation of lactose by *Streptococcus thermophilus*. In the present author's laboratory, experiments with *Streptococcus thermophilus* have resulted in more rapid fermentation of lactose than of glucose. Fermentation of glucose was always slow or slight and in one instance a fermentation of lactose was nearly complete before glucose was fermented at all.

Hoff-Jørgensen *et al.* (12) have shown that one strain resembling *Lactobacillus bulgaricus* will grow in a medium containing glucose plus lactose but will not grow in the same medium containing glucose only.

The evidence for the direct fermentation of maltose and certain other carbohydrates has been reviewed by Leibowitz and Hestrin (4).

Experiments in this laboratory in which attempts have been made to isolate a lactase from extracts of lactose-fermenting yeasts have yielded disappointing results. At different times either no lactase was found or the quantity present was negligible. In the latter case the presence of lactase was a transitory phenomenon. These experiments induced us to undertake the work reported here.

The evidence for the direct fermentation of lactose, as presented by other workers, is persuasive and is based primarily on the kinetics of the fermentation. However, certain objections are difficult to invalidate.

It is the purpose of this paper to present data showing the relative rates of fermentation by diverse types of lactose-fermenting yeasts, as well as to present new and more definite proof of the direct fermentation of lactose by lactose-fermenting yeasts.

EXPERIMENTAL

Methods

The organisms employed in this work were *Torulopsis kefir* 149, *Saccharomyces lactis* 131, *Torula lactosa* 168, *Zygosaccharomyces lactis* 90, *Torulopsis sphaerica* 13, *Saccharomyces anamensis* 145,¹ type F 93,² *Candida pseudotropicalis* 32, *Mycotorula lactis* 130, *Saccharomyces fragilis* 15, and *Torulopsis cremoris* 2.

¹ This strain, received from the Lister Institute, is obviously incorrectly designated. According to Stelling-Dekker (13) *Saccharomyces anamensis* should ferment maltose but not lactose.

² Type F appears to be a variety of *Saccharomyces fragilis* unable to ferment inulin.

According to Diddens and Lodder (14), *Mycotorula lactis* is a representative of *Candida pseudotropicalis* and *Torula lactosa* a strain of *Candida pseudotropicalis* var. *lactosa*. These organisms are as representative a group of lactose-fermenting yeasts and yeast-like fungi as it was possible to obtain.

Strains 145, 93, 32, 130, 2, and 15 were incubated at 37° and strains 131, 149, 90, 13, and 168 at 30°. These temperatures are within the optimal range for both growth and fermentation.

The source cultures used for inoculating the experimental fermentations were grown on agar slopes of the following composition: Bacto-tryptone 0.5 per cent, Bacto-yeast extract 0.3 per cent, KH_2PO_4 0.2 per cent, agar 2 per cent, and glucose 2 per cent. Except for one type of experiment, which will be described later, the stock cultures had been grown for many transfers (five times weekly for nearly a year) on the above medium containing *glucose* as the sole carbohydrate. Since many yeasts are capable of adapting themselves to the fermentation of certain carbohydrates, particularly galactose, glucose was chosen in order to avoid the complications of adaptation. The importance and significance of this point will be discussed later.

The inocula for the fermentations were grown on agar slopes for 48 hours. The cells were washed from the surface and suspended in sterile distilled water. The suspension was shaken mechanically and uniform aliquots in any given experiment were inoculated into the fermentation flasks which contained a medium of the following composition: Bacto-tryptone 0.5 per cent, Bacto-yeast extract 0.3 per cent, KH_2PO_4 0.2 per cent, 85 per cent lactic acid 0.04 per cent, and the appropriate carbohydrates or mixtures of carbohydrates at the desired concentrations. The pH of this medium was approximately 5.1 and remained constant during autoclaving. The flasks were of 125 ml. capacity and contained 78 gm. of the fermentation medium which had been dispensed by an automatic pipette. The flasks were plugged with cotton and autoclaved no longer than 5 minutes at 15 pounds steam pressure in order to avoid decomposition of the sugars.

Glucose and galactose were Pfanstiehl products with specific rotations of +52.5° and +80.5°, respectively. The lactose was recrystallized from C.P. lactose and treated with norit A six times. The specific rotation of the lactose was +52.5° at equilibrium. These values were obtained independently by two analysts and are in agreement with accepted values (15).

The progress of the fermentations was followed by a direct determination of the residual sugar by polarimetric analysis. 3 times the normal weight of the sugar were taken in each case and clarified with lead subacetate. The normal weights were accepted as 32.248 gm. for glucose, 32.857 gm. for lactose, and 21.429 gm. for galactose. All polarimetric

analyses were conducted at 20°. Results of many analyses were usually within 0.05 per cent of the known concentrations of the various carbohydrates. For the analyses of mixtures of glucose and galactose, the primary fermentation with the lactose-fermenting yeast was terminated by momentary autoclaving. After cooling, the flask was reinoculated with *Saccharomyces chevalieri*, American Type Culture Collection No. 9804. This yeast ferments glucose but not lactose or galactose, and it does not become adapted to the fermentation of galactose even after long periods of time. Thus the residual galactose was determined directly, and the residual glucose in a mixture of glucose and galactose was determined by

TABLE I
Fermentation of Glucose, Galactose, and Lactose by Representative Lactose-Fermenting Yeasts

Organism	Sugar fermented*		
	Glucose	Galactose	Lactose
	per cent	per cent	per cent
<i>T. kefir</i>	0.42, 1.02, 1.22, 3.03	0.20, 0.29, 0.81, 0.93	0.42, 0.80, 1.49, 1.88
<i>S. lactis</i>	0.88, 1.55, 3.38, 4.15	0.99, 2.18, 4.08, 4.54	0.84, 1.46, 2.74, 3.26
<i>T. lactosa</i>	0.70, 1.48, 2.92, 3.54	0.63, 1.20, 2.67, 3.19	0.70, 1.46, 2.61, 3.50
<i>Z. lactis</i>	0.59, 1.05, 2.15, 2.53	0.21, 0.47, 1.00, 1.50	0.53, 0.91, 2.47, 2.57
<i>T. sphaerica</i>	0.45, 1.02, 2.00, 2.74	0.35, 0.43, 1.39, 2.57	0.53, 0.87, 1.95, 2.56
<i>S. anamensis</i>	0.88, 1.66, 3.27, 4.09	0.82, 1.59, 2.95, 3.73	0.84, 1.36, 3.20, 3.20
Type F	1.12, 2.00, 3.27, 4.09	0.97, 1.78, 3.06, 3.43	1.19, 2.31, 3.71, 3.70
<i>C. pseudo-tropicalis</i>	0.88, 1.44, 2.85, 3.27	0.92, 1.73, 2.81, 3.19	0.88, 1.50, 2.57, 3.29
<i>M. lactis</i>	0.59, 1.12, 2.15, 2.53	0.67, 1.30, 2.01, 2.24	0.90, 1.50, 2.55, 2.85
<i>S. fragilis</i>	0.84, 1.44, 2.25, 2.57	0.85, 1.43, 2.29, 2.49	1.08, 1.53, 2.61, 3.30
<i>T. cremoris</i>	0.96, 2.07, 4.01	0.18, 1.56, 2.94	0.01, 1.97, 4.18

* The figures represent values for 18, 26, 42, and 50 hours, respectively, of elapsed fermentation time.

difference. Appropriate uninoculated controls were included so that the correct constants for calculating percentages of sugar from the observed rotations could be determined.

Results

In Table I are summarized the results of experiments which show the relative attenuation of the individual sugars, glucose, galactose, and lactose by eleven representative lactose-fermenting yeasts. The initial concentration of each sugar was 5 per cent. If the whole course of the fermentations is considered, the average rate of fermentation as determined by the method of tangents is as great, or greater, for lactose as for glucose in the case of

several organisms. The average rates, expressed as the percentage of sugar fermented per hour, in the case of glucose and lactose, respectively, were 0.077 and 0.079 for *Torula lactosa*, 0.088 and 0.098 for type F, 0.065 and 0.064 for *Candida pseudotropicalis*, 0.052 and 0.057 for *Mycotorula lactis*, 0.052 and 0.068 for *Saccharomyces fragilis*, and 0.10 and 0.174 for *Torulopsis cremoris*. Moreover the maximal rates of a fermentation were greater for lactose than for glucose with all the above organisms and also with *Zygosaccharomyces lactis* and *Saccharomyces anamensis*. This must mean that lactose was being dissimilated more rapidly than glucose, at least during one stage of the fermentations. And this occurred even with organisms that could be considered glucose-adapted strains.

A striking result, as Table I shows, was the relative slowness with which galactose was fermented by most of the strains. Only four of the eleven strains fermented galactose at average rates, throughout the entire fermentation, that were equal to or in excess of those for glucose. Of these four strains only two, *Saccharomyces lactis* and *S. fragilis*, consistently fermented galactose as well as they fermented glucose, or better, at nearly all intervals of the fermentations, and only one strain, *S. lactis*, fermented galactose more readily than either glucose or lactose for the entire duration of the fermentations. With this organism the average rates for glucose, galactose, and lactose, respectively, were 0.086, 0.099, 0.067; the maximal rates were 0.114, 0.149, and 0.080. Thus, in only one of eleven instances can it be said that galactose was fermented fairly consistently at a rate greatly in excess of that of lactose.

One peculiar feature of the galactose fermentation is the occasional instance in which galactose is fermented more slowly than either glucose or lactose during the greater part of the fermentation, until suddenly galactose begins to be fermented in an explosive-like manner (see the results with *Torulopsis sphaerica*, Table I). The rate of fermentation may change abruptly from a value of 0.047 to 0.147 for a relatively short period. The author is not referring to changes from the lag or induction period to the logarithmic phase of fermentation, but rather to abrupt changes of rate within the logarithmic phase itself. Obviously, such an abrupt change would affect the average rate as well as the maximal rate of fermentation and make it appear from the figures that galactose is fermented better than it really is during most of the fermentation period.

The results shown in Table I do not describe the course of a complete fermentation in which all the initial sugar is finally fermented. Fig. 1, however, does depict the entire courses of the fermentations of glucose, galactose, and lactose by *Torulopsis cremoris*. During the 14 to 26 hour interval, the slopes of the glucose and lactose curves were practically identical at a value of 0.142, and the slope of the galactose curve was only

0.104. In the 26 to 48 hour interval the slope of the glucose curve was 0.120, that of the lactose curve was 0.132, and the rate for galactose was considerably less at a value of 0.091. The glucose fermentation was complete at about 50 hours and the lactose fermentation at about 48 hours, but galactose was not completely fermented even after 62 hours. At this time 20 per cent of the initial concentration of galactose remained unfermented. This experiment was repeated with a strain "adapted" to galactose through a series of twenty-seven transfers. The results were substantially the same as above. The slight differences noted were within the limits of experimental variation.

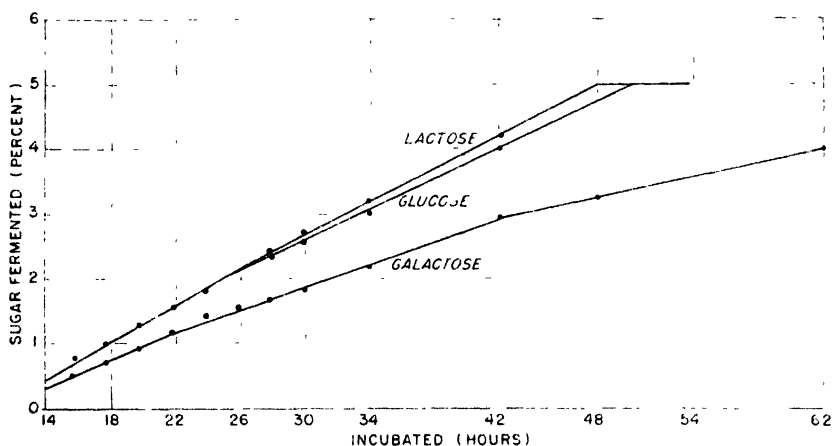


FIG. 1. Fermentation of glucose, galactose, and lactose, individually, by *Torulopsis cremoris* 2.

The experiments already described were conducted with media containing 5 per cent of carbohydrate. It is true that most of these strains, except *Torulopsis cremoris*, are affected adversely by the accumulation of end-products, particularly ethanol. Preliminary experiments showed that 3 per cent of the sugar did not result in an inhibitory concentration of end-products. Since lactose hydrate should hydrolyze to yield 1 mole each of glucose and galactose, experiments were performed to compare the fermentation of 3 per cent lactose with equivalent mixtures of glucose and galactose. All the strains were tested in such experiments. The data for strain 93 shown in Table II are representative to varying degrees of the results obtained with all strains, except strain 131, and the special case of strain 149. As Table II shows, lactose was fermented completely, while 0.66 per cent of sugar, or 44 per cent of the initial concentration of galactose, remained unfermented in the mixture. The glucose in the mixture was

fermented completely. Although, as pointed out previously, galactose alone is fermented fairly well by a few strains, this sugar is fermented poorly in the presence of glucose by all strains except strain 131, presumably because of a preferential glucose fermentation.

Strain 131, unlike the other organisms, fermented galactose well, indeed so well that galactose was fermented in preference to glucose in a mixture of these sugars. The results in Table III show that galactose was fermented completely within 50 hours, whereas 0.81 per cent of glucose or 54

TABLE II
Fermentation of Glucose, Lactose, and Equivalent Mixture of Glucose and Galactose by Type F 93

Fermentation time	Sugar fermented			
	Glucose	Lactose	Glucose-galactose	
	per cent	per cent	per cent	per cent
hrs				
19	1.19	1.10	0.76	0.27
27	1.89	1.98	1.50	0.52
43	3.00	3.00		0.84
50				1.23
67				1.31

TABLE III
Fermentation of Glucose, Lactose, and Equivalent Mixture of Glucose and Galactose by Saccharomyces lactis 131

Fermentation time	Sugar fermented			
	Glucose	Lactose	Glucose-galactose	
	per cent	per cent	per cent	per cent
hrs.				
19	1.19	0.80	0.61	0.27
50	2.22	2.52	0.69	1.50
67	2.89	2.63	0.72	1.50

per cent of the initial concentration remained unfermented. Originally it was thought that, since this exceptional strain ferments galactose alone at a rate superior to that of glucose alone, a lactase enzyme might be operative in the fermentation of lactose. However, the results do not confirm this expectancy because lactose was fermented significantly faster throughout most of the fermentation period than an equivalent mixture of glucose and galactose.

Results with *Torulopsis kefir* 149 were the most interesting and definite. The ability of this strain to ferment glucose, galactose, lactose, and equivalent mixtures of glucose and galactose was tested in repeated experiments.

The results for a glucose-adapted strain are shown in Table IV. Here it is seen that the fermentation of glucose in the 3 per cent control sample was complete within 43 hours and the fermentation of 3 per cent lactose within 67 hours. The glucose in a mixture of glucose and galactose was completely fermented within 43 hours. *The galactose (1.5 per cent) in this mixture was not fermented at all.*

The results just described for *Torulopsis kefir* 149 were obtained with a glucose-adapted strain. It is important to know (1) whether this organism can be adapted to ferment galactose, and (2) whether the galactose-adapted strain behaves similarly to the glucose-adapted strain. Accordingly, the glucose-adapted strain was passed through media containing glucose, galactose, and lactose, each singly, by means of massive inocula through twenty-seven transfers. These adapted strains were then tested in the individual sugars and in a mixture of glucose and galactose equivalent to

TABLE IV
Fermentation of Glucose, Lactose, and Equivalent Mixture of Glucose and Galactose by Torulopsis kefir 149

Fermentation time <i>hrs.</i>	Sugar fermented			
	Glucose	Lactose	Glucose-galactose	
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
19	0.52	0.15	0.35	0
27	1.93	1.13	1.43	0
43	3.00	1.57	1.50	0
50		2.08		
67		3.00		

the concentration of lactose. These results are shown in Table V. The fermentation of 3 per cent glucose was complete in the cases of all the adapted strains. Galactose, itself, at an initial concentration of 3 per cent was fermented slightly (only 14.33 per cent of the initial concentration) within 48 hours by the glucose-adapted strain. Galactose at an initial concentration of 1.5 per cent was not fermented within 31 hours and only 4.66 per cent of the initial concentration was fermented within 48 hours. This difference in the slight fermentation of galactose, dependent on the initial concentration, was sometimes encountered. There is no apparent explanation for this. But again the glucose-adapted strain fermented lactose individually, but not galactose in a mixture of glucose and galactose, through a period of at least 48 hours.

The galactose-adapted strain fermented galactose and adaptation took place. Despite the fact that galactose was now being fermented by the galactose-adapted strain, lactose was completely fermented within 57 hours,

while 0.20 per cent of galactose (or 13.33 per cent of the initial concentration) remained unfermented in a mixture of glucose and galactose at the end of 71 hours. Furthermore, lactose was fermented at a faster average rate (0.070) than was the case with equivalent mixtures of glucose and galactose (0.051). Near the end of the fermentation when lactose was fermented completely, the rate for lactose during a 2 hour period was 0.090, while the rate for glucose-galactose during this same interval and an additional period of 14 hours (at the end of which there remained 0.20 per cent of residual sugar) was only 0.019.

TABLE V
*Fermentation of Glucose, Galactose, and Lactose by Adapted Strains of
Torulopsis kefir 149*

Substrate	Sugar fermented*		
	Glucose strain	Galactose strain	Lactose strain
	per cent	per cent	per cent
Glucose (3%)	0.76, 1.92, 3.00	0.39, 1.36, 2.55, 3.00	0.69, 1.70, 2.96, 3.00
Galactose (3%)	0.07, 0.18, 0.43, 0.66, 0.80	0.16, 0.57, 1.48, 2.14, 2.59	0.25, 0.75, 1.80, 2.36, 2.82
Lactose (3%)	0.43, 0.98, 1.66, 2.35, 2.71	0.40, 1.19, 2.24, 2.82, 3.00	0.47, 1.27, 2.35, 3.00
Glucose (1.5%)-galactose (1.5%)	1.18, 1.50, 1.50	0.48, 1.05, 1.80, 2.50, 2.80	0.48, 1.25, 2.02, 2.75, 3.00
Glucose (1.5%)	1.18, 1.50	0.39, 1.32, 1.50	0.71, 1.50
Galactose (1.5%)	0, 0, 0.07, 0.22, 0.27	0.10, 0.55, 1.25, 1.50	0.22, 0.67, 1.00, 1.50

* The figures represent values for 23, 31, 48, 55, and 71 hours, respectively, of elapsed fermentation time.

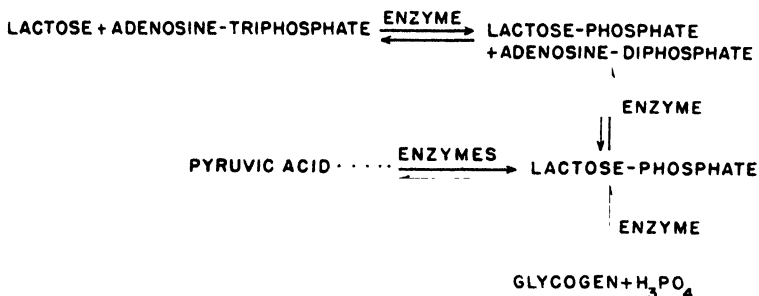
The lactose-adapted strain, as was to be expected, fermented lactose at a faster rate than either of the other two adapted strains. The fermentation of lactose was complete within 55 hours. At this time 0.25 per cent of sugar remained unfermented in the glucose-galactose mixture. But the striking fact was not that the lactose-adapted strain fermented lactose faster than a mixture of glucose and galactose, but rather that a lactose-adapted strain should, in the absence of any contact with galactose, simultaneously adapt to galactose, for the lactose-adapted strain fermented completely 1.5 per cent of galactose and also 3.0 per cent of galactose more rapidly in each case than did the galactose-adapted strain itself. This is a puzzling result for which the author has no ready explanation.

DISCUSSION

All of the organisms studied fermented lactose at a faster rate than that at which they fermented a mixture of glucose and galactose. In addition, however, it must be remembered that one glucose-adapted strain fermented lactose completely but did not ferment galactose in a mixture of glucose and galactose. It is difficult on the basis of this direct result to argue that a preliminary hydrolysis of lactose in a fermentation is necessary.

The reason that lactose is fermented faster than a mixture of glucose and galactose lies in the fact that galactose, itself, normally is fermented with relative difficulty. On the basis of present knowledge it is only possible to speculate as to the reason for the slowness of the galactose fermentation.

The importance of phosphorus in carbohydrate metabolism is now well established. If preliminary hydrolysis of lactose in a fermentation does



not occur, then it is necessary to postulate a reaction analogous to that of Price, Cori, and Colowick (16). This is shown in the accompanying scheme.

The reaction scheme may not be exactly as shown, but it is not unreasonable to assume the formation of phosphoric esters with disaccharides. Doudoroff *et al.* (17) have already demonstrated the phosphorolysis of sucrose and related compounds in fermentation by *Pseudomonas saccharophila*.

The existence of galactose-1-phosphate has been demonstrated by Kosterlitz (18). The author cannot explain the simultaneous adaptation of a lactose-adapted strain to galactose. The possibility of the existence of a lactose-phosphate ester has not yet been investigated. Until these gaps in our knowledge are bridged, it is not possible to argue with assurance concerning the mechanism of lactose fermentation. Considerable work remains to be done.

SUMMARY

The lactose-fermenting yeasts adapted to glucose, galactose, or lactose fermented lactose faster than they fermented an equivalent mixture of

glucose and galactose. Normally this is because of a relatively weak galactose fermentation.

One lactose-adapted strain, in the absence of any contact with galactose, simultaneously became adapted to the fermentation of galactose.

One glucose-adapted strain fermented lactose completely, while it did not ferment galactose in a mixture of glucose and galactose.

Enzymatic hydrolysis of lactose is unnecessary for the fermentation of lactose. Fermentation may be "direct" by means of phosphorolysis and phosphorylation.

The significance of these results in relation to modern schemes of carbohydrate metabolism is discussed.

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CARBONIC ANHYDRASE IN THE CENTRAL NERVOUS SYSTEM OF THE DEVELOPING FETUS*

By WINIFRED ASHBY AND ELLEN BUTLER

(From the Blackburn Laboratory, Saint Elizabeths Hospital, Washington)

(Received for publication, April 24, 1948)

Well defined patterns of quantitative distribution of carbonic anhydrase have been reported in the central nervous system of man and of animals (1, 2). Two reports have been made on deviations from the normal pattern of distribution in man incident to mental disease (3, 4). As part of an effort to elucidate the significance of this enzyme in the central nervous system, a study of its fetal distribution was undertaken.

Studies by various workers have been reported on oxygen uptake and on increase in individual enzymes with maturation of the central nervous system. These were made both upon the young of animals immature at birth and upon fetuses of species comparatively mature when born.

Measurements of O_2 uptake by the central nervous system in puppies from birth to 7 weeks of age, made by Himwich and Fazekas (5), show an increase in metabolic rate proceeding from the medulla to the caudate nucleus and approaching the pattern of the adult in which the respiratory metabolism is found to be higher in the newer phylogenetic layers than in the older. Adult oxygen consumption was not attained. Similar findings are reported by Tyler and van Harreveld for the rat (6).

Studies on the distribution of the enzymes choline esterase, cytochrome oxidase, succinic dehydrogenase, and succinic oxidase show a similar rostral movement in attainment of maximum content. In the human fetus obtained by hysterotomies and hysterectomies Youngstrom (7) reports a rapid increase in choline esterase content of the cord, medulla, and mid-brain from the 56th to the 121st day of fetal age. In the basal ganglia and cerebrum there was a slight increase up to 4 months, when a precipitous increase occurred. A similar study was made by Nachmansohn (8) in fetal goats. Flexner, Flexner, and Straus (9, 10) studied the increases, in the cerebral cortex only, of cytochrome oxidase, succinic dehydrogenase, and succinic oxidase with growth of the fetal pig. Between the 60th day and birth at 114 days there were two increases in these enzymes which correlated with morphologic changes in the cortical tissue.

Studies by Meldrum and Roughton (11) on the development of carbonic anhydrase in the blood of fetal goats and those of Stevenson (12) on the

* This work was supported by funds contributed by the Supreme Council, 33° Scottish Rite Masons of the Northern Jurisdiction, United States of America.

blood of premature infants indicate a late appearance of any considerable quantity of that enzyme.

EXPERIMENTAL

Technique—In this study carbonic anhydrase was determined by the colorimetric method and correction was made for the enzyme contained in the red blood cells remaining in the tissue as previously reported (13). Seven fetal calves were obtained incident to the slaughter of the hospital herd of cows for beef; there was no delay in obtaining specimens. A calf was sacrificed immediately after birth for this study. The fetal ages were obtained from the breeding records, except those of Fetuses II and V for which an estimate of age was made on the basis of development.

TABLE I
Increase of Carbonic Anhydrase in Blood of Fetus (Cattle)

Age	Packed corpuscles	Age	Packed corpuscles
days	units per cc.	days	units per cc.
96	100	244	615
113	222	257	678
206	244	267	526
241	588	275	597
241	519	At birth	640
243	625	Adult	700

Gestation period 275 to 283 days.

Specimens from the central nervous system of the infants were obtained from the Gallinger Municipal Hospital. Five of these infants were born prematurely and were underweight, but were free of evidence of disease. A full term child which weighed 8 pounds was born alive, but did not survive. Autopsies were performed within 48 hours after death; the bodies were in the meantime appropriately stored with respect to temperature.

Results with Fetal Cattle—Incident to the determination of the correction to be made for carbonic anhydrase in the red blood cells of the tissue, the enzyme content of packed corpuscles was determined. It was found that in the blood of the youngest fetus studied, aged 96 days, there was a considerable amount of the enzyme present. This increased to the 8th month, after which it remained at approximately the same level until birth. Data on ten fetuses of known ages are given in Table I.

Carbonic anhydrase contents of various areas from the central nervous system of five fetuses ranging in age from 96 to approximately 240 days are charted in Fig. 1. No carbonic anhydrase was demonstrable in the cere-

brum of any of these animals after that present in the blood of the tissue was accounted for. In a sample consisting of a mixture of cerebellum and brain stem of Fetus I, aged 96 days, a slight tissue enzyme content may have

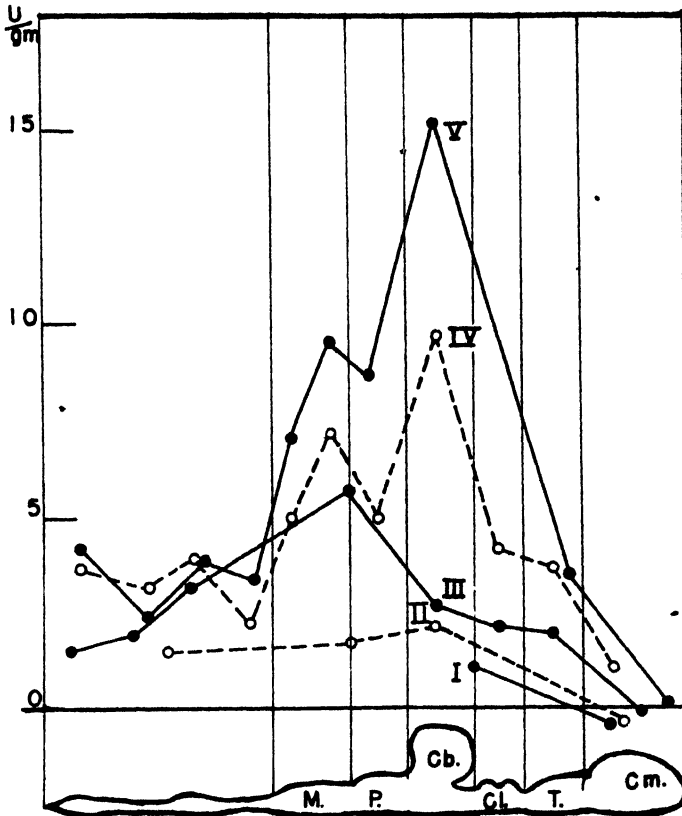


FIG. 1. Rostral progression of distribution of carbonic anhydrase in the central nervous system of the cattle fetus from the 96th day of gestation to an estimated 8 months. Fetus I, length 7½ inches, weight ½ pound; Fetus II, 18 inches, 7½ pounds; Fetus III, 21 inches, 13½ pounds; Fetus IV, 29 inches, 31 pounds; Fetus V, 33 inches, 34 pounds. Units of carbonic anhydrase per gm. of wet tissue are plotted as the ordinates. M. = medulla, P. = pons, Cb. = cerebellum, Cl. = colliculi, T. = thalamus, Cm. = cerebrum.

been present; the cord could not be obtained. In Fetus II the enzyme was definitely present in the cord, in the combined pons and medulla, and in the cerebellum, although the amount was so slight that differences could not be estimated. The cord of Fetus III, aged 113 days, contained an amount of enzyme approaching that found in the adult; the amount in the combined medulla and pons was increased, but that of the cerebellum and colliculi

was still low. In Fetus IV, 206 days old, the content of the cerebellum had surpassed that of the medulla and pons and had attained the prominence seen in the adult; there were considerable increases in the colliculi and thalamus. Fetus V, approximately 240 days old, showed about the same amount in the cord as did Fetus IV, and a further increase in more rostral parts.

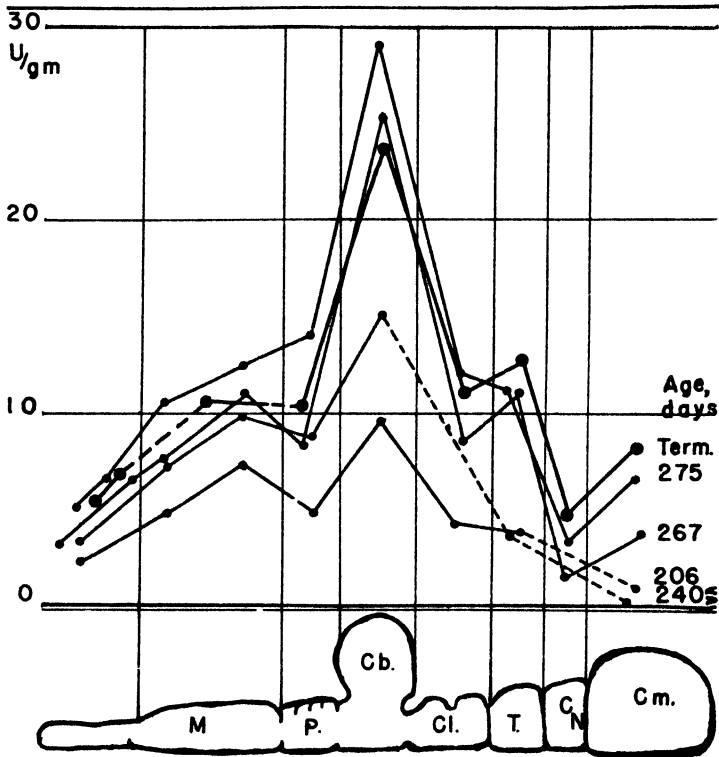


FIG. 2. Similarities in the pattern of distribution of carbonic anhydrase in the central nervous system of the developing cattle fetus from the 206th day of gestation to term. Where a comparable area is omitted a broken line is used. CN. = nucleus caudatus; other letters as in the legend to Fig. 1.

Two additional fetuses were subsequently obtained which were nearer to their full growth. One of these was 267 days old and the other 275 days. The gestation period is 275 to 286 days. In these fetuses carbonic anhydrase was found in the cerebrum; in the 267 day-old fetus the amount was 3.7 units per gm. and in the 275 day-old fetus it was 6.7 units per gm., as against 8 units for the new-born. Except for this appearance of carbonic anhydrase in the fetal cerebrum shortly before term, the four older fetuses

studied, beginning with that aged 206 days, show a remarkably constant pattern of quantitative distribution, which is like that of the new-born. The data illustrating this consistency of pattern are charted in Fig. 2.

At the time of birth the adult pattern was established, although at a lower level. In Fig. 3 the data obtained from the study of Fetus V, of the

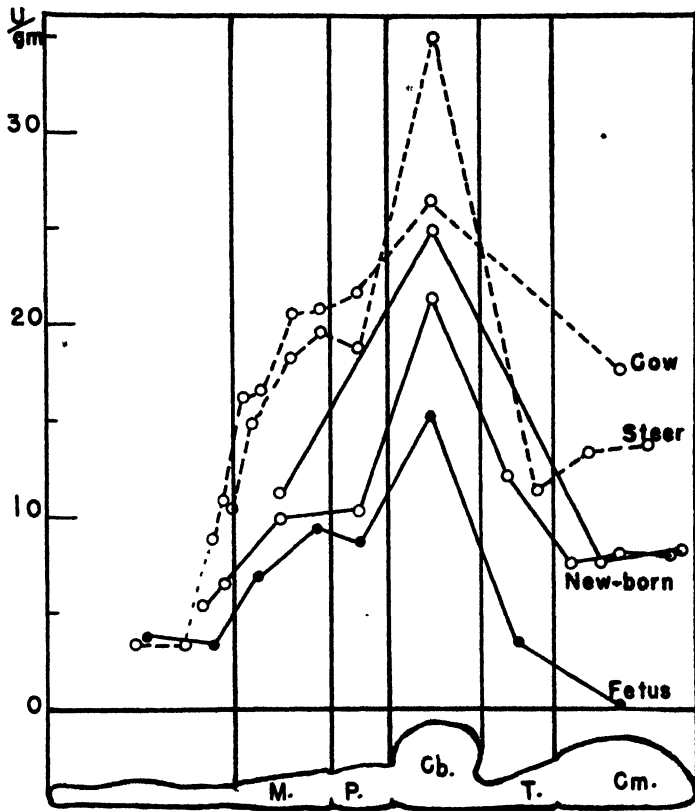


FIG. 3. Approximation to the adult pattern of distribution of carbonic anhydrase in the central nervous system in the 8 month cattle fetus, a new-born calf, and a calf a few days old as compared with an 18 month-old steer and a 3 year-old cow. Letters as in Fig. 1.

calf which was sacrificed immediately after birth, and of a calf which died of an infection soon after birth are compared with data from a steer and a cow. It is seen that when the enzyme content of the same areas was measured the relationships between them were very generally the same within the error of the technique. The outstanding exception is that of the relationship between the thalamus and the cerebrum in the new-born and the steer.

TABLE II
Data on Human Infants

Fetus No.	Weight	Menstrual age	Carbonic anhydrase	Notes
		mos.	units per cc. red blood cells	
I		6	<30	Separation of placenta
II			<30	Still-born
III	2 lbs., 2 oz.	6	<30	Lived 5 hrs., lungs atelectatic
IV	2 " 13 "	7*	<30	Breathed, died
V	2 " 3 "	8*	<30	Difficult labor
VI	8 "	9	145	Died at birth
Adult			600	

* History of full term, but the ages given were estimated by the neurologist on the basis of the development of the brain.

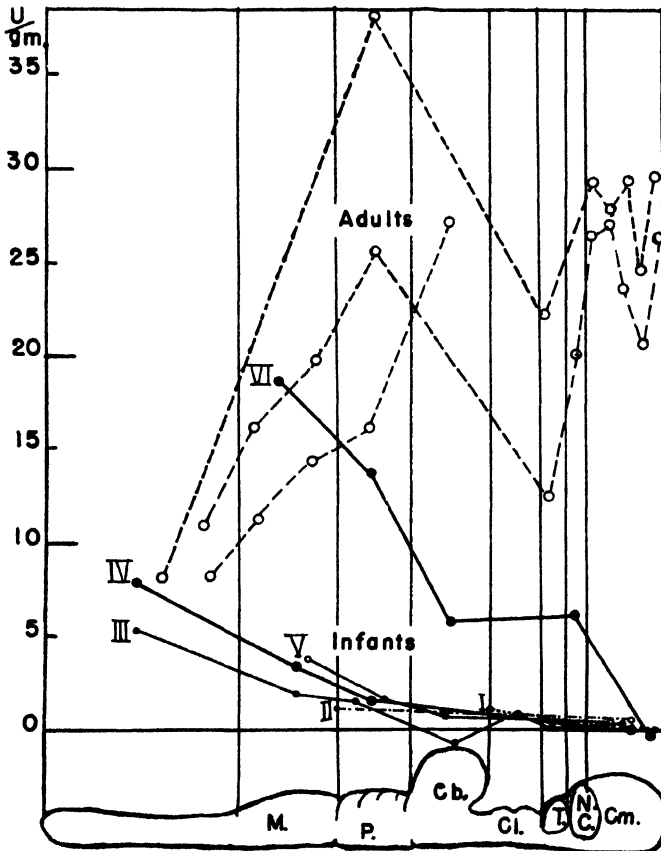


FIG. 4. Delayed approach to the human adult pattern of carbonic anhydrase distribution in the central nervous system of Fetuses I to VI. Letters as in Figs. 1 and 2.

In the new-born the thalamus had attained the enzyme level found in the steer, whereas the lag in development of carbonic anhydrase content in the cerebrum of the new-born resulted in a reversal of the relationship seen in the older animal.

Data from Human Infants—Clinical data concerning the infants studied are given in Table II. In Fig. 4 are charted the carbonic anhydrase findings for five premature infants and for the one born at 9 months. In contrast to the preceding findings in cattle, the pattern of distribution at birth does not parallel that of the adult, but tends to stand in reverse order, the phylogenetically older regions of the central nervous system having the higher content. No carbonic anhydrase was found in the cerebrum. In the premature infants the spinal cord has the maximum content, approaching that found in the adult, but the more rostral areas showed little development of the enzyme. In the 8 pound infant of 9 months the content of the medulla had developed to adult proportions, but that of the pons had definitely lagged. In the human adult the content of the pons has always been found to be higher than that of the medulla. In this infant the contents of the cerebellum and caudate nucleus were still further from adult proportions.

DISCUSSION

Unlike studies of other enzyme increases in the central nervous system of the fetus, the study of carbonic anhydrase shows a complete absence of the enzyme from the pallium until shortly before birth, although as with other enzymes reported, a progressive rostral increase, emerging towards the adult pattern of distribution, is indicated.

With the exception of an absence of this enzyme from the pallium, the fetal calf at 206 days showed a distribution closely paralleling the pattern found in the central nervous system of adult cattle. There was evidence of a caudal-rostral approach to this pattern in the 113 day fetus. Although more than half of the adult cerebral content was found in the calf at birth, the fetus taken 1 to 8 days before birth, depending upon what its gestation period would have been, showed a content of 83 per cent of that of the new-born and the cerebrum of a fetus 12 days younger contained 50 per cent. This would suggest a steep increase of the enzyme within the last month of gestation, with a preceding absence from the cerebrum coincident with a fairly high level in the fetal blood.

Similarly the human fetus showed a rostral increase in carbonic anhydrase with development. However, even with a content in the cord approaching that of the adult level, little or none was found in the higher centers of the premature infant and, in contrast to the new-born calf, no enzyme was found in the pallium of the full term infant, although it was

present in the cerebellum and caudate nucleus. Whether this absence of carbonic anhydrase from the cerebrum at birth is normal for the human new-born, being associated with its greater immaturity, or is a symptom of a lowered physiologic efficiency which resulted in the failure of this child to survive birth, remains to be determined. But in either event, because of its late appearance in the cerebrum, carbonic anhydrase would seem to fall in a different category from that of the other enzymes so far studied in the fetal brain.

SUMMARY

Study of the distribution of carbonic anhydrase in the central nervous system with development of the normal fetus of cattle has indicated the following: (1) a progressive caudal to rostral increase in the enzyme approaching the adult pattern; (2) in contrast to other enzymes studied in the fetal central nervous system an absence of the enzyme from the cerebrum until late in fetal development; (3) from the 7th month to birth, except for its absence in the pallium, a clean cut repetition of the pattern of relative content of the enzyme with an approach in magnitude to the level found in the adult. An early appearance of the enzyme was found in the blood of fetal cattle, in contrast to the finding of Meldrum and Roughton for fetal goats.

In the human premature and full term infants the data indicate the following: (1) a caudal to rostral increase in the enzyme with increase in menstrual age; (2) a less complete approach to the adult pattern of distribution in the full term infant than in the 6 month cattle fetus; (3) no carbonic anhydrase in the cerebrum at birth.

The possibility that the absence of carbonic anhydrase from the cerebrum of the full term human infant may be abnormal is considered.

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UTILIZATION BY THE RAT OF 3-HYDROXYANTHRANILIC ACID AS A SUBSTITUTE FOR NICOTINAMIDE*

By HERSCHEL K. MITCHELL, JOSEPH F. NYC, AND RAY D. OWEN

(From the Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena)

(Received for publication, May 8, 1948)

Experiments by a number of workers have established the fact that addition of tryptophan to the diet results in increased synthesis of nicotinic acid by a number of mammals (1-7).

As suggested by Rosen, Huff, and Perlzweig (2), the facts indicate that tryptophan is a natural precursor of nicotinic acid. Recently a series of papers from this laboratory (8-10) has presented evidence that the mold *Neurospora* is able to convert tryptophan to nicotinic acid, and that kynurenine and 3-hydroxyanthranilic acid are intermediates in the conversion. Although kynurenine is a well known metabolic product of tryptophan in animals, 3-hydroxyanthranilic acid was not known chemically or biochemically prior to its recent synthesis (9, 10). The natural occurrence of 3-hydroxyanthranilic acid was indicated by its presence as a trimethyl derivative (damascenine) in the seeds of *Nigella*. More recently it has been shown by Bonner (11) to be identical with a precursor of nicotinic acid that is accumulated by a mutant of *Neurospora*.

The present work is concerned with the question of whether or not 3-hydroxyanthranilic acid will replace nicotinamide for the rat, as it does for *Neurospora*. A preliminary report by Bonner (11) has indicated that the compound does not replace nicotinamide or tryptophan for rat growth. However, in view of the instability of the substance it seemed likely to the authors that the requirement by rats might be rather high due to loss through oxidation and possibly methylation in the animals. Consequently, in the experiments to be described, 3-hydroxyanthranilic acid has been fed at a moderate and at a high level as compared to the amount of nicotinamide given.

EXPERIMENTAL

Basal Diet—The basal diet used in these experiments is very similar to Diet 1, Group 5, as described by Krehl *et al.* (4). The composition of the diet is given in Table I.

Experimental Procedure—Five litters (thirty-six rats) were obtained from

* This work was supported by grants from the Williams-Waterman Fund for the Combat of Dietary Diseases and from the Rockefeller Foundation.

a cross of two inbred strains. One of these, an agouti Irish line, is from stocks provided in 1946 by Dr. W. F. Dunning (Wayne University). The other is an albino line designated "M. I." by Dr. A. B. Chapman (Univer-

TABLE I
Basal Diet

Corn-meal (Albers, white)	4 kg.
Casein (Casein Company of America, vitamin-free)	1.5 "
Sucrose	7.8 "
Corn oil	7.8 "
Cystine	15 gm
Choline chloride	1 "
Thiamine " ..	20 mg.
Riboflavin .	30 "
Pyridoxine hydrochloride	25 "
Calcium pantothenate	200 "
Inositol	1 gm.
Biotin	0.2 mg.
Folic acid	5 "
Vitamin K	100 "
Salt mixture*.	400 gm.
Vitamins A, D, E†	2 drops per rat per wk.

* Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935)

† A mixture of 200 drops of corn oil, 100 drops of halibut liver oil, and 100 mg. of α -tocopherol.

TABLE II
Weights of Rats at Beginning and End of Experiment

Group No	No. of rats	Initial weight		Final weight	
		Range	Average	Range	Average
		gm.	gm.	gm.	gm.
I	8	29-46	40	39-79	58
II	6	33-50	42	63-91	85
III	6	27-50	37	57-105	73
IV	6	25-48	37	43-79	56
V	6	42-47	44	67-107	91

sity of Wisconsin) from whom the stock was obtained in 1946. Hybrids of this type presumably combine a high degree of genetic uniformity with a considerable amount of vigor. The basal diet was supplied to mothers and litters when the young were 2 weeks old. At 21 days the young rats were placed in small cages in pairs of males or females. After 3 more days

on the basal ration, the animals were placed in the following five groups: Group I, basal diet, four males, six females; Group II, basal diet plus 2 mg. of nicotinamide per 100 gm. of ration, two males, four females; Group III, basal diet plus 100 mg. of tryptophan per 100 gm. of ration, four males, two females; Group IV, basal diet plus 10 mg. of 3-hydroxyanthranilic acid per 100 gm. of ration, four males, four females; Group V, basal diet plus

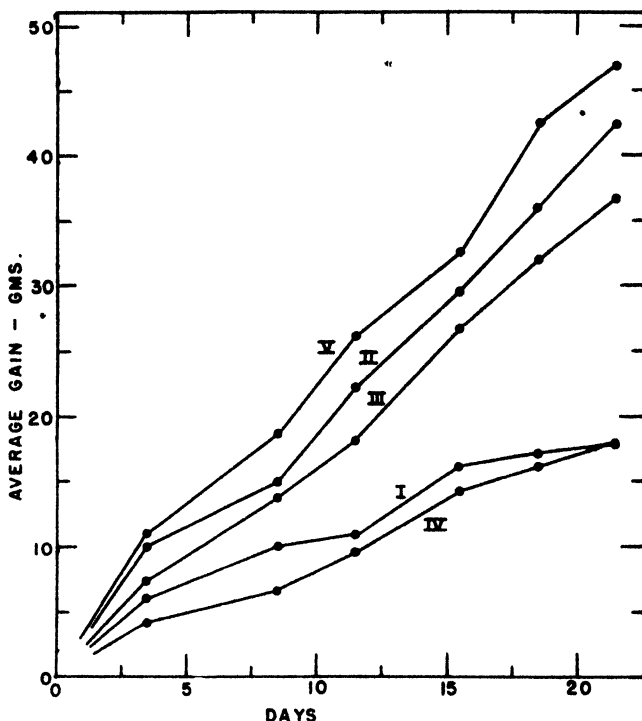


FIG. 1. Average total gain in rats on the basal ration (Group I), nicotinamide (Group II), tryptophan (Group III), 3-hydroxyanthranilic acid-low (Group IV), and 3-hydroxyanthranilic acid-high (Group V).

100 mg. of 3-hydroxyanthranilic acid per 100 gm. of ration, four males, two females.

In all cases food and water were given *ad libitum*, fresh daily. Although no special precautions were taken to prevent coprophagy, cages, feeders, and water bottles were cleaned frequently. The animals were raised in a constant temperature room at 25°.

Growth Results—A general summary of the weights of rats in the various groups is given in Table II. It should be noted that all of the rats of the five litters were used. Except for pairing with respect to sex, rats were

placed in the groups at random with no attempt at equalizing the groups with respect to weights at the beginning of the experiment. No significant relationship was noted between initial weight or sex of an individual animal and the total weight gain during the period of the experiment.

Data on the average total gain of the groups of rats during the course of the experiment are given in Fig. 1.

In addition to the experiment on dietary 3-hydroxyanthranilic acid, four animals were given the compound by subcutaneous injections. 9 days after the feeding experiment was begun, two rats were taken from Group I and two from Group IV. These animals were then kept on the basal ration.

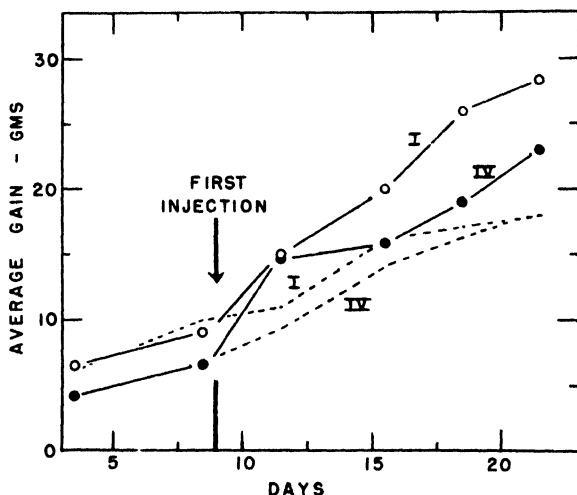


FIG 2. Average gain in weight by rats following subcutaneous injections of 3-hydroxyanthranilic acid. The dotted lines represent the average increase in weight in the groups from which the pairs of rats were taken.

10 mg. of the compound suspended in 0.5 ml. of saline were given per rat at 9, 12, and 16 days. Growth performance of these animals is summarized in Fig. 2.

DISCUSSION

It seems clear from the data presented that 3-hydroxyanthranilic acid will replace nicotinamide in the diet of rats. The requirement is evidently somewhat less than that for tryptophan but considerably more than that for nicotinamide. Hydroxyanthranilic acid is rather unstable toward oxidation in the pH range above 6, and, in addition, methylation is to be expected in biological systems. In view of these considerations the relatively high requirement for the compound as compared to nicotinamide is

not surprising. It is worthy of note that 3-methoxyanthranilic acid is significantly inhibitory to the conversion of tryptophan to nicotinic acid by *Neurospora* (unpublished). A similar effect may exist in animals if this compound is a metabolic product of 3-hydroxyanthranilic acid.

The data on the growth effect of subcutaneously injected 3-hydroxyanthranilic acid are too limited to be more than suggestive. It is worthy of note that the injected animals received very nearly the same quantity of the compound as the animals of Group IV did in the diet during the same period of time (12½ days). In spite of this fact the injected animals increased in total growth, while the growth of Group IV was slightly inferior to that of the controls (Fig. 2). Although more extensive information along this line might contribute toward answering the question of whether intestinal flora or animal tissues are responsible for the growth effect of 3-hydroxyanthranilic acid, it is doubtful that growth experiments can settle the question.

It has been shown that *Neurospora* is able to convert tryptophan, kynurenine, and 3-hydroxyanthranilic acid to nicotinic acid (8, 9). The fact that the rat utilizes 3-hydroxyanthranilic acid as a substitute for nicotinamide suggests that in this animal the conversion of tryptophan to nicotinamide follows a pathway similar to that in *Neurospora*.

SUMMARY

1. Data are presented to show that 3-hydroxyanthranilic acid can be substituted for nicotinamide or tryptophan in promoting growth of rats.

2. The requirement for 3-hydroxyanthranilic acid is considerably higher than that for nicotinamide when the compounds are given by mouth. A limited amount of data indicates a lower requirement when the compound is injected subcutaneously.

3. Results indicate that the mechanism of biosynthesis of nicotinamide in the rat is similar to that in the mold *Neurospora*.

The authors wish to express their appreciation to Mr. G. L. Merchant of Merck and Company for assistance in obtaining some of the constituents of the basal diet. The corn-meal was furnished by the Albers Milling Company, Oakland, California.

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ALKALINE PHOSPHATASE IN THE INTESTINAL LYMPH OF THE RAT

By EUNICE V. FLOCK AND JESSE L. BOLLMAN

(From the Division of Experimental Medicine, Mayo Foundation, Rochester, Minnesota)

(Received for publication, April 26, 1948)

A decrease of the alkaline phosphatase activity of plasma has been observed during fasting in rats, guinea pigs, and dogs (1-5). An increase from the fasting level has been produced in dogs by the administration of a carbohydrate meal (6) and in rats by the administration of a fat meal (2, 5, 7). These observations, as well as the fact that the intestinal mucosa has the highest content of alkaline phosphatase of any tissue in the body, have led to the suggestion that the intestinal mucosa may be a source of the alkaline phosphatase of plasma (6-8). Analysis of the intestinal lymph provides more direct evidence for this function of the intestinal mucosa. We have found that the alkaline phosphatase activity of lymph from the small intestine decreases with fasting and increases after feeding to higher levels than does that of the plasma. Moreover, when the intestinal lymph is collected continuously and thus diverted from its normal passage into the blood stream, the concentration of this enzyme in the blood decreases in the fed rat to very low levels.

Methods

Male rats of the Sprague-Dawley strain weighing approximately 200 gm. were used in these studies. They were maintained on either Friskies or a fat-free diet prior to operation. A cannula was introduced into an intestinal lymphatic trunk in the mesentery just prior to its entrance into the cisterna chyli. All operative procedures were carried out with the rats under ether anesthesia. The cannula was of polyethylene tubing of 1 mm. outer diameter and 0.6 mm. internal diameter. Since the ligature placed about the lymphatic trunk would include other branches, it was felt that all of the lymph from the small intestine was obtained in most preparations. The rats were placed in small cages with free access to water. Saline solution was given daily by subcutaneous injection. The lymph was collected continuously and at intervals was analyzed for inorganic phosphate by the method of Fiske and Subbarow (9). For alkaline phosphatase activity 0.1 or 0.2 ml. aliquots were incubated with 5 ml. of substrate, essentially by the method of Bodansky (10). In the later experiments the activator, $MgCl_2$, in 0.2 per cent concentration, was included in Bodansky's substrate. The unit of phosphatase activity used in Tables II to VIII is

defined as the amount liberating 1 mg. of P from the substrate when hydrolysis proceeds for 1 hour at 37°. Similar analyses were made of plasma from blood removed by cardiac puncture.

As a check on the completeness of the collection of intestinal lymph, studies were also made of rats in which a polyvinyl chloride tubing (11) had been introduced into the thoracic duct.

Generally the first meal was administered on the morning after the day of operation. In most of the experiments a comparison was made of the effect of 7.5 ml. of the fat-free diet (Table I) given by stomach tube and of the effect of 1 ml. of corn oil administered just prior to the 7.5 ml. of fat-free

TABLE I
Diets Used for Administration by Stomach Tube

	Fat-free diet	13 per cent fat diet
	gm.	gm.
Corn oil		13
Cascien	20	6
Starch	24	40
Dextrin	20	20
Sucrose	19	19
Salt mixture (12)	4	2
Vitamin mixture*	0.069	0.069
Oleum percomorphum		0.5
	ml.	ml.
Water	85	85
Total volume.	135	153
Caloric value, calories per ml.	2	3

* Thiamine hydrochloride 20 mg., riboflavin 20 mg., nicotinic acid 40 mg., pyridoxine 20 mg., calcium pantothenate 40 mg., choline chloride 500 mg., and 2-methylnaphthoquinone 50 mg. Total, 690 mg. per kilo diet.

diet. In later experiments the corn oil and the fat-free diet were emulsified in the Waring blender prior to administration. The effect of a 13 per cent fat meal, only, was studied in the rats with fistulas of the thoracic duct.

Results

There was a definite increase of the alkaline phosphatase activity of the intestinal lymph of rats receiving the fat-free meal (Fig. 1). In these rats the control sample of lymph was obtained by an overnight collection. After a temporary decrease of concentration shortly after the administration of the meal, largely owing to an increased flow of lymph, there was an increase to a peak at from 6 to 12 hours, with a return toward normal during the last half of the 24 hour period. Considerable variation was noted in the

maximal levels of phosphatase found in these rats. This variation was probably due to variations of rate of gastric emptying.

The group of rats which received 1 ml. of corn oil in addition to the fat-free meal but without preliminary mixing showed increases of phos-

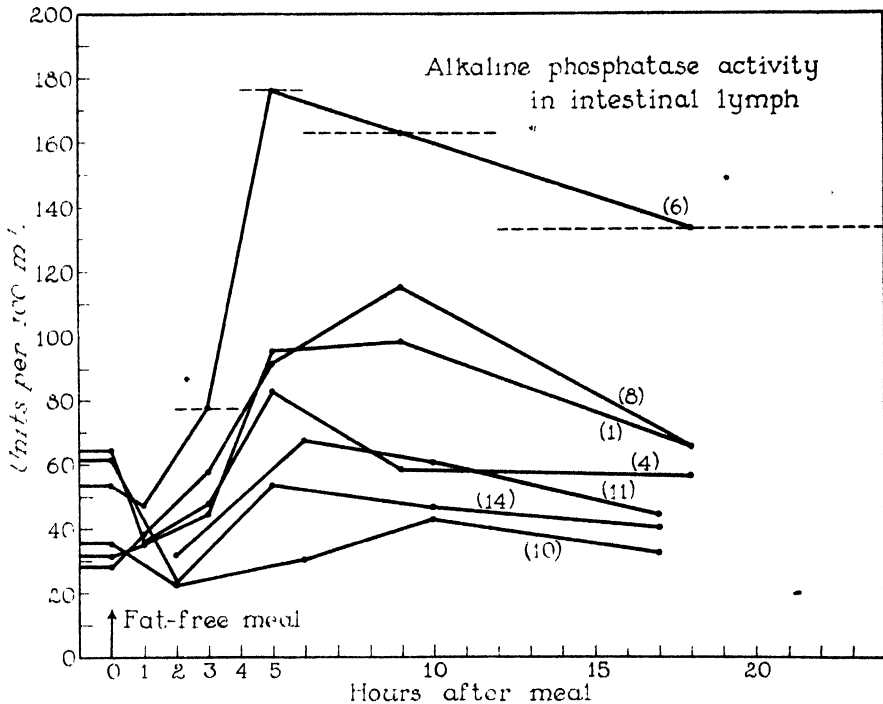


FIG. 1. The effect of a fat-free meal on the alkaline phosphatase activity of intestinal lymph. All rats had been maintained on the Friskies diet up to the time of operation, except Rat 4, which had been shifted to a fat-free diet 1 week before the operation. The period of collection of the other samples is shown by the broken line, the actual value found being plotted in the center of the time interval and representing the average value for the period. The number of each rat is given in parentheses for comparison with Table II.

phatase activity of the intestinal lymph (Fig. 2) which were quite similar to those found in the rats which had received the fat-free meal only.

The effect of Mg^{++} as activator on the alkaline phosphatase activity of the lymph was measured in five of the rats shown in Figs. 1 and 2. On the average there was a 42.5 per cent increase in the peak values in the presence of Mg^{++} . As a typical example the curves for alkaline phosphatase in the lymph of Rat 12 with and without Mg^{++} in the substrate are shown in Fig. 3.

The range of values for the total volume of lymph collected during a 24 hour period and for total units of phosphatase in this volume of lymph was essentially the same both in the group which received the fat-free meal only and in the group which received fat in addition (Table II).

After the 24 hour period of lymph collection just described each rat received the opposite test meal. The lymph was collected for 6 or 12

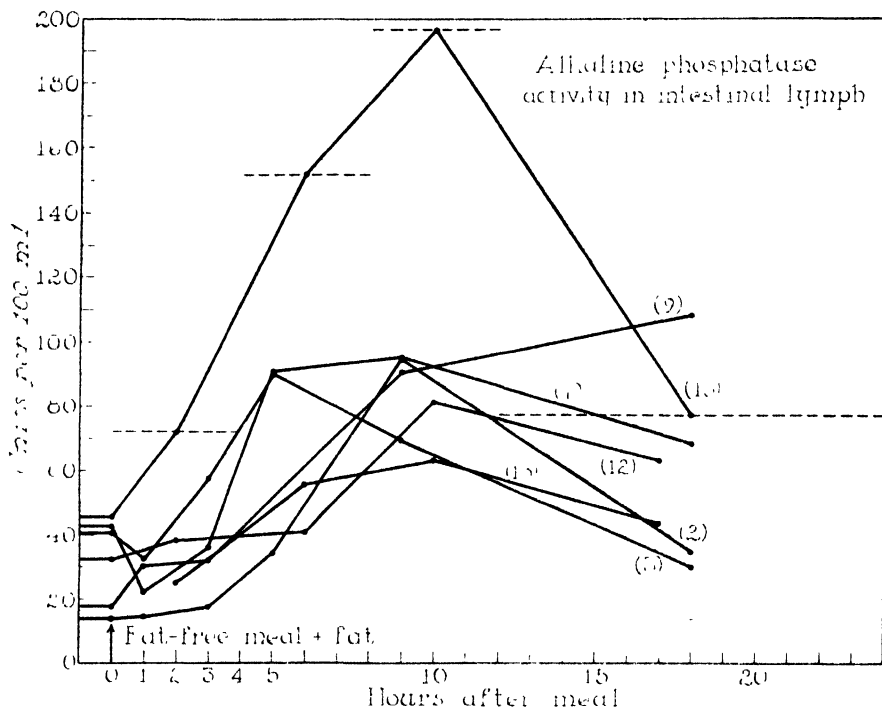


FIG. 2. The effect of feeding 1 ml. of corn oil by stomach tube, followed by the same fat-free meal as in Fig. 1. All rats had been maintained on the Friskies diet up to the time of operation.

hours and then the blood was taken for analysis (Table III). The total time that the lymph was collected in these rats varied from 52 to 56 hours.

In another series of rats, a 2 hour control sample of lymph was collected on the next morning after operation, a meal was given, and the lymph was collected for three periods of 4 hours each. At the end of this time, when the alkaline phosphatase level in the lymph was high, the blood was taken for comparison (Table IV). Increase in units of enzyme activity were found per 100 ml. of lymph and per volume of lymph collected after both meals, but the increases were much larger in the four rats which received the fat-free meal plus fat. The generally higher level of values found in

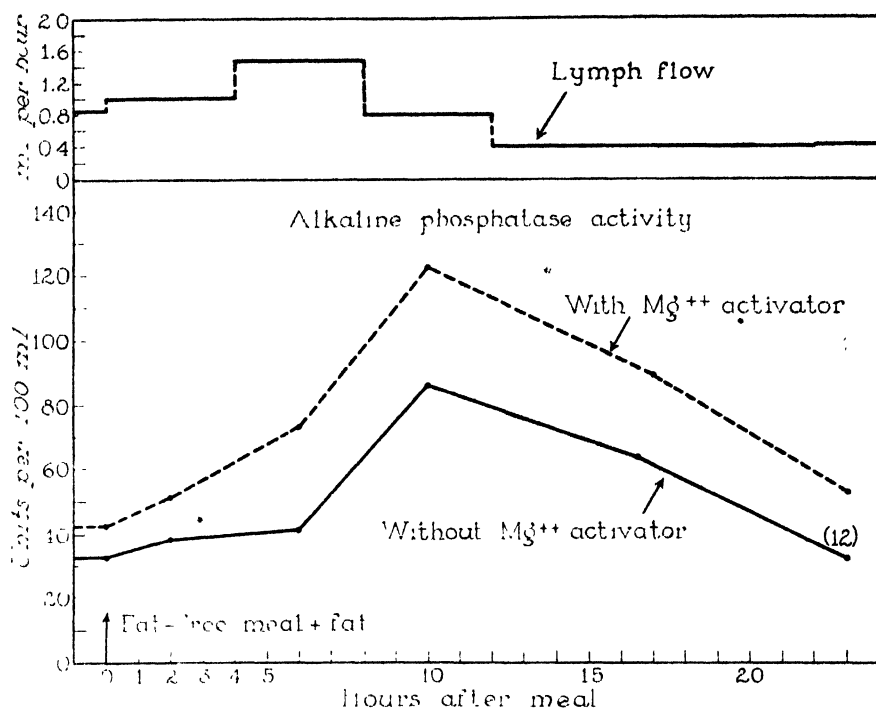


FIG. 3. The average hourly flow of intestinal lymph in Rat 12. The alkaline phosphatase activity shown with the solid line is the same as shown in Fig. 2. The level of this activity found with Mg^{++} for an activator is shown with the broken line.

TABLE II

Volume and Alkaline Phosphatase Activity of Intestinal Lymph over 24 Hour Period

Fat-free meal			Fat-free meal + fat		
Rat No.	Volume	Alkaline phosphatase activity, units per day	Rat No.	Volume	Alkaline phosphatase activity, units per day
	ml.			ml.	
1	16.4	10.0	2	9.2	4.0
6*	13.2	14.5	7*	12.6	6.3
8	14.3	10.8	12	17.3	9.1
10	11.6	3.6	13	21.3	9.7
11	13.6	6.2	15	13.9	14.2
14	15.9	5.5			

* These two rats had been maintained on a fat-free diet for 1 week prior to the operation; all others had been on the Friskies diet. None were fasted prior to operation.

this group was in contrast also to that of the rats receiving the fat-free plus fat meal shown in Fig. 2. This result may be related to the fact that in the observations shown in Table IV the fat was emulsified at the time of feeding.

The alkaline phosphatase activity of the plasma of rats from which the lymph had been collected continuously for 52 to 56 hours was very low (Table III). Since the rats had been fed 6 or 12 hours previously, the content of this enzyme was at a high level in the intestinal lymph. Also in the group of rats in which the plasma was analyzed after the lymph had

TABLE III

Comparison of Alkaline Phosphatase Activity of Intestinal Lymph and Plasma after 52 to 56 Hours of Lymph Collection and 6 or 12 Hours after Meal

Rat No.	Alkaline phosphatase activity, units per 100 ml.			
	Intestinal lymph		Plasma	
	Activator		Activator	
	None	Mg ⁺⁺	None	Mg ⁺⁺
4	132		5.8	
6*	189		4.8	
7*†	56.7		4.8	
10	27.1	39.6	19.7	32.1
11	52.1	85.4	2.3	4.9
12†	42.6	56.4	5.7	15.9
13†	51.6	74.8	2.5	5.6
15†	47.1	69.6	2.4	6.2

* These two rats had been on a fat-free diet for 1 week before the operation; all others had been on a Friskies diet.

† These four rats received a fat-free meal; others received the fat-free meal plus fat.

been collected continuously for a shorter time interval, 32 to 35 hours, similar low values for the alkaline phosphatase of the plasma were found (Table V). Analyses of plasma from rats in which the intestinal lymphatic fistulas were unsatisfactory, that is, had ceased to function at some time during the experiment, are included in Table V. In most of these rats, collateral lymphatic channels allowed the lymph to flow into the cisterna. Such rats which had received the fat-free meal had 13.2 to 21.1 units of alkaline phosphatase per 100 ml. of plasma, while those which had received the fat-free meal plus fat had 33.9 and 64.8 units. These values are higher than the range of 5.1 to 11.6 units found in the plasma of the rats in which the lymph flow through the fistulas was continuous. Intact rats studied at the same interval after feeding had from 20.8 to 44.2 units of phosphatase

per 100 ml. of plasma if they had received the fat-free meal and from 50.4 to 68.6 units if they had received the fat-free meal plus fat. Thus it is seen that, when the lymph is flowing continuously from the fistula, the level of alkaline phosphatase in the plasma decreases to very low levels, but, if the

TABLE IV

Changes in Alkaline Phosphatase Activity of Intestinal Lymph during a 12 Hour Period Following Meal

Rat No.	Meal	Alkaline phosphatase activity with Mg ⁺⁺			
		Control*	Hrs. after meal		
			0-4	4-8	8-12
		units per 100 ml.	units per 100 ml.	units per 100 ml.	units per 100 ml.
19	Fat-free	37.4	14.9	43.8	55.5
20		18.5	9.4	19.4	57.8
24		54.2	30.7	51.6	73.8
25		47.8	21.6	50.2	67.5
26	" + fat†	45.0	50.8	78.2	128
27		47.2	42.0	153	178
30‡		110.6	58.4	223	255
32‡		62.0	43.6	197	232
		units per hr.	units per hr.	units per hr.	units per hr.
19	Fat-free	0.15	0.12	0.18	0.26
20		0.02	0.10	0.11	0.22
24		0.11	0.15	0.19	0.31
25		0.22	0.23	0.25	0.30
26	" + fat	0.10	0.71	0.70	0.54
27		0.10	0.49	0.69	0.98
30		0.16	1.34	1.28	1.28
32		0.17	0.78	0.98	1.11

* 2 hour collection from 22 to 24 hours after operation.

† The entire meal was homogenized in the Waring blender prior to tube feeding, in contrast to the procedure used in Table II, when 1 cc. of the corn oil was given by stomach tube, followed by 7.5 cc. of the fat-free meal.

‡ Maintained on a fat-free diet for 3 days prior to operation without fasting. All others had been on a Friskies diet up to a 24 hour fast prior to the operation.

fistula ceases to work, the level in the plasma returns toward normal values.

The effect of fat on the level of plasma phosphatase noted in the rats with unsatisfactory lymph flow and in the intact rats after tube feeding with and without fat is also evident in rats eating *ad libitum* the fat-free diet or the diet of Friskies (4.5 per cent fat) (Table VI). The unfasted rats eating Friskies had 79.7 units of plasma alkaline phosphatase and with fasting this decreased to low levels in 24 to 48 hours. Unfasted rats eating the

fat-free diet had only 18.2 units of this enzyme, and the effect of fasting was less.

TABLE V

Alkaline Phosphatase Activity 12 Hours after Feeding, and after 32 to 35 Hours of Continuous Collection of Intestinal Lymph

Meal	Alkaline phosphatase activity with Mg^{++} , units per 100 ml.				
	Plasma				Intestinal lymph
	Intact rats		Lymph flow unsatisfactory	Lymph flow continuous	
Fat-free	29.4	44.2	18.8	9.0	55.5
	35.9*	36.8	13.2	10.8	57.8
	23.4*	23.6	21.1	7.8	73.8
	20.8*	31.6		5.8	67.5
" + fat	57.8		64.8	5.1	128
	68.6*		33.9	6.2	178
	50.4*			11.6	255
	66.5*			7.2	232

* The rats were maintained on the fat-free diet for 3 days prior to the final meal; the others were maintained on the Friskies diet and then fasted 24 hours before operation. The lymph sample was collected from 8 to 12 hours after the meal. The values for plasma at the end of this period are shown in the adjacent column.

TABLE VI

Alkaline Phosphatase Activity of Plasma of Normal Rats

No. of rats	Diet	Duration of fasting	Inorganic phosphate, mg. per 100 ml.	Alkaline phosphatase activity, units per 100 ml.	
				Activator	
				Mg^{++}	None
		hrs.			
10	Friskies	0	7.3 ± 0.2	79.7 ± 6.1	
8	"	24	7.2 ± 3.6	20.2 ± 3.6	
9	"	48	6.4 ± 0.2	16.5 ± 1.8	
6*	Fat-free	0	5.5 ± 0.2	18.2 ± 1.2	13.9 ± 2.0
6*	"	24	5.6 ± 0.1		11.4 ± 1.1
6*	"	48	5.4 ± 0.3		11.1 ± 0.9

* Two rats in each group had been on the fat-free diet for from 7 to 9 days, others from 47 to 58 days. The figures after the $\pm$ represent the standard error of the mean.

In an attempt to see just how rapidly the enzyme does decrease in the plasma, additional rats were studied in which the meal was administered

TABLE VII

Alkaline Phosphatase Activity of Intestinal Lymph and Plasma after Short Periods of Collection

Meal	Alkaline phosphatase activity with Mg ⁺⁺ , units per 100 ml.			
	Plasma			Intestinal lymph
	Intact rats	Sham operation*	Lymph flow continuous	
24 hrs. after operation				
13% fat	46.8	46.8 "	33.5	151
	40.1	35.7	24.8	137
	34.0		8.6	188
	35.7		9.5	188
6 hrs. after operation				
Friskies			11.8	120
			19.3	44.8
			18.0	73.1
			30.0	67.7
			31.8	92.9
			20.0	18.0

* The operation included dissection of the lymphatics and was fully as extensive as the operation in which a cannula was introduced.

TABLE VIII

Alkaline Phosphatase Activity of Thoracic Duct Lymph 5 to 6 Hours after Feeding, and after 1 to 4 Days of Continuous Collection of Lymph

Meal	Alkaline phosphatase activity with Mg^{++} , units per 100 ml.		
	Plasma		Thoracic duct lymph
	Lymph flow unsatisfactory	Lymph flow continuous	
13% fat*	73.6	9.7	124
	24.9	10.2	203
	69.3	6.2	162
		8.0	136
Fat-free		6.7	152
		5.5	43
" + fat		3.2	48
	18.7		
	18.6		

* The rats were maintained by tube feeding of the 13 per cent fat diet twice daily (60 calories total). The lymph sample in the last column was a 5 or 6 hour specimen. The values for plasma phosphatase taken at the end of this period are in the adjacent column

12 hours after operation and the lymph and plasma were compared 12 hours after the meal (Table VII). As early as 24 hours after collection of lymph had been begun, the units of phosphatase were low in the plasma. Another group of rats was operated on without a period of fasting, and the lymph was collected for only 6 hours. In these rats the enzyme had decreased in the plasma but not to as low a level as in the rats from which the lymph had been collected for a longer period.

Low levels of alkaline phosphatase were found also in the plasma of rats from which the lymph had been collected continuously for 1 to 4 days from the thoracic duct (Table VIII). In three rats in which the fistula had ceased to function during the experiment higher values for plasma alkaline phosphatase were found, two of which approached normal values.

Comment

Relatively little is known about the chemical composition of intestinal lymph, largely because of the difficulties of collection. However, with the technique developed in this laboratory for introducing plastic tubing into the intestinal lymphatic trunk, it has become possible to collect such lymph continuously for several days from animals which are otherwise normal. The alkaline phosphatase of intestinal lymph has been found to decrease with fasting but usually not to as low levels as that of the plasma; the enzyme increases after feeding to much higher levels than in the plasma. The increase occurs after a fat-free meal or the same meal plus fat, but the increase may be greater after the latter meal. Previously Weil and Russell (2) and Dalgaard (5) have stated that the increase of the level of this enzyme in the plasma is obtained in the rat only after a fat meal. The quantity of the fat-free meal administered by Weil and Russell was much smaller than in our experiments. Gould (7) had found very high levels of alkaline phosphatase activity in the plasma of rats maintained on a very high fat diet. In our experiments we have found higher values for plasma phosphatase in rats receiving fat in addition to the fat-free meal than in those receiving the fat-free meal only, but an increase was found after either meal.

The observation of a higher content of alkaline phosphatase in the intestinal lymph than in the plasma shows that the small intestine is a source of this enzyme for the plasma. Additional evidence is provided by the observation that the content in the plasma decreases to very low levels when the flow of lymph from the fistula is continuous and returns toward normal levels when the flow of lymph ceases. The finding of low levels of alkaline phosphatase in the plasma of rats when all the lymph was collected from the thoracic duct as well as from the intestinal lymphatics was to be expected, since all the intestinal lymph was drained in either case.

It appears that alkaline phosphatase is supplied to the plasma from the small intestine by way of the lymph in the rat.

SUMMARY

The alkaline phosphatase of intestinal lymph of the rat decreases with fasting and increases after either a fat-free meal or the same meal plus fat. Higher levels of the enzyme may be found after a fat meal, however, in both lymph and plasma than after a fat-free meal. The concentration of the enzyme in the intestinal or thoracic duct lymph is much greater after feeding than in the plasma of intact rats after feeding.

The concentration of plasma alkaline phosphatase is greatly reduced when all the intestinal lymph is collected continuously either directly or through the thoracic duct. It appears that alkaline phosphatase is supplied to the plasma from the small intestine by way of the lymph in the rat.

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ISOLATION OF 17-HYDROXYCORTICOSTERONE FROM THE URINE IN A CASE OF CUSHING'S SYNDROME ASSOCIATED WITH SEVERE DIABETES MELLITUS

By HAROLD L. MASON

(From the Division of Biochemistry, Mayo Foundation, Rochester, Minnesota)

AND RANDALL G. SPRAGUE

(From the Division of Medicine, Mayo Clinic, Rochester, Minnesota)

(Received for publication, May 4, 1948)

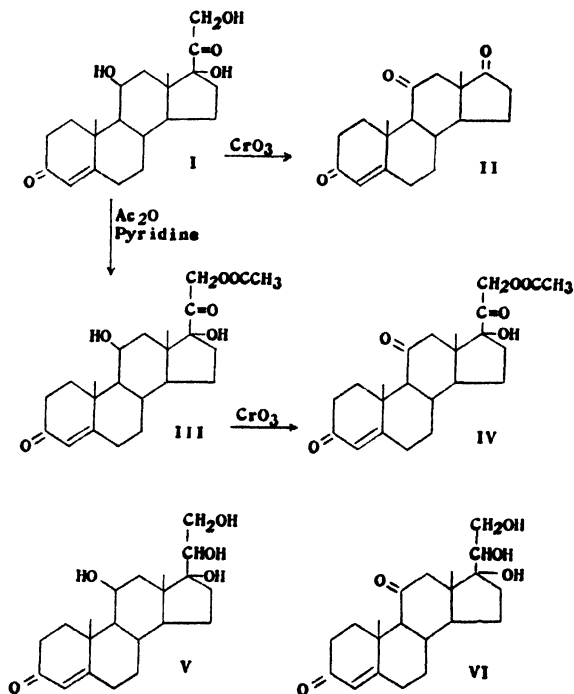
Many of the features of Cushing's syndrome are those that would be expected to result from an oversupply of adrenal cortical hormones. Indeed, Albright (1) has attributed the development of this syndrome to overproduction by the adrenal cortex of the carbohydrate-active hormones (S hormone), while Kenyon (2) and Kepler and his associates (3) have explained some of the features of the syndrome, including the diabetes, by the action of these steroids, and other features by the action of other adrenal steroids. Evidence in support of these views has been found in an increased excretion of carbohydrate-active material in the urine as measured by stimulation of glycogenesis in the liver of the adrenalectomized animal (4), and also as measured by less specific chemical methods (5). It was therefore a matter of considerable interest to attempt to isolate the active material from the urine when a case of Cushing's syndrome presented itself in which the associated diabetes mellitus was unusually severe. In this case the excretion of corticosteroid-like substances varied between 14 and 19 mg. (in terms of 11-dehydrocorticosterone) per day. The patient, a boy 14 years of age, was found at surgical exploration, and later at necropsy, to have hyperplastic adrenal cortices.¹

The method for the determination of corticosteroid-like substances was based on the procedure of Lowenstein, Corcoran, and Page (6), in which the amount of formaldehyde generated by the action of periodic acid is determined. The amount of formaldehyde is taken as a measure of the amount of the groups $\text{—COCH}_2\text{OH}$ and $\text{—CHOHCH}_2\text{OH}$, the former group being an essential characteristic of the cortical hormones. In order to avoid interference by the steroid residues and impurities the formaldehyde was distilled prior to the colorimetric procedure.

The urine was collected daily, acidified to pH 1, and extracted with chloroform after standing 1 to 3 days at room temperature. The extracts were pooled and worked up when enough had been accumulated. It was

¹ A report of this case, including metabolic studies, will be presented elsewhere.

decided to use the method of solvent partition that had been used successfully for the isolation of hormones from extracts of the adrenal cortex (7). To this end the chloroform was removed, the residue was taken up in benzene, and the benzene solution was extracted fifteen times with an equal volume of water. The combined aqueous extracts were concentrated to about 250 ml. and extracted with chloroform. Concentration of the chloroform solution to a small volume led to separation of crystals which were filtered out and thoroughly washed with chloroform. A total of 292



mg. of crude crystals was thus obtained from a 25 day collection of urine and this crude material yielded 191 mg. of purified 17-hydroxycorticosterone (I).² This amount of purified hormone corresponds to an average of 7.6 mg. per day.

The crude crystals melted at 200–205°. After crystallization from absolute alcohol the melting point was 213–215° and $[\alpha]_D^{25} = +167^\circ \pm 3^\circ$. The melting point was not changed by successive crystallizations from acetone and methanol. These properties, together with the formation of a red 2,4-dinitrophenylhydrazone, the development of a yellow-green fluorescence on treatment with concentrated sulfuric acid, and the prompt

² The roman numerals refer to the structural formulas in the figure.

reduction of alkaline silver in the cold, suggested the likelihood that the substance was 17-hydroxycorticosterone (8-10). Examination of the absorption in the ultraviolet with a Beckman spectrophotometer revealed an absorption maximum at $242\text{ m}\mu$ ($\epsilon = 15,800$) which is characteristic of an α,β -unsaturated ketone group. The structure of the compound was firmly established by oxidation to adrenosterone (II) (8, 9) and by oxidation of the acetate (III) to 17-hydroxy-11-dehydrocorticosterone acetate (IV) (10).

The only other structures that would possibly give one or more of these results are Reichstein's (11) Substances E (V) and U (VI). Both have the α,β -unsaturated ketone group and both would yield adrenosterone on oxidation with chromic acid. The former compound, however, melts at $126-127^\circ$ and $[\alpha]_D^{25} = +87^\circ$. The properties of Substance U more nearly agree with those of the urinary compound. It melts at 208° and $[\alpha]_D^{25} = +178.5^\circ$ (acetone). It is excluded from further consideration, however, by the fact that it forms the 20,21-diacetate (m.p. $252-253^\circ$) under the conditions used for acetylation of the urinary compound. This diacetate would not be susceptible to oxidation to 17-hydroxy-11-dehydrocorticosterone acetate. Indeed, Reichstein and von Euw (11) converted the diacetate of Substance E to the diacetate of Substance U by oxidation with chromic acid. Substances E and U are further excluded by their failure to reduce alkaline silver solution at room temperature.

Dr. D. J. Ingle kindly consented to assay the compound by his muscle-work test and reported that it contained 9.66 units per mg. Dr. Ingle stated that this value is within 15 per cent of the value recently obtained in his laboratory for 17-hydroxycorticosterone.

The isolation of appreciable quantities of 17-hydroxycorticosterone from the urine in this case is in accord with the view that at least some of the manifestations of Cushing's syndrome are primarily the result of an overproduction of those adrenal cortical hormones which are active in carbohydrate metabolism. Although there is no way of estimating the amount of 17-hydroxycorticosterone that was produced by the hyperplastic adrenal cortices, it seems probable that the amount excreted in the urine could not have been more than 10 per cent of the amount produced, and very likely was much less. This estimate is based on the low recovery of corticosteroids in the urine when 11-dehydrocorticosterone and 17-hydroxy 11-dehydrocorticosterone were given to human subjects (12).

The presence in this case of severe diabetes which was refractory to insulin appeared to be analogous to the insulin-resistant hyperglycemia and glycosuria induced by Ingle, Sheppard, Evans, and Kuizenga (13) in rats by administration of 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone.

EXPERIMENTAL

Extraction and Isolation—The urine was collected in periods of 24 hours. The volume of urine excreted during 24 hours was between 3 and 4 liters. After removal of a small aliquot for various determinations the remainder was acidified to pH 1 with concentrated HCl and allowed to stand 1 to 3 days at room temperature. It was then extracted four times with 0.15 volume of chloroform that had been distilled over K_2CO_3 . The combined chloroform extracts were concentrated under reduced pressure and at a bath temperature of 50° to 20 to 30 ml. Several such extracts were combined, diluted to 100 to 200 ml. with chloroform, washed three times with 10 ml. of cold 0.1 N NaOH for each 100 ml. of chloroform solution, and then washed three times with a volume of water equal to that of the NaOH. The chloroform was distilled under reduced pressure at a bath temperature of 50°. The residues were pooled in a little wet chloroform and stored in the refrigerator.

The pool of material obtained during a 16 day period was evaporated to dryness under the conditions just described and the residue was extracted with successive portions of redistilled thiophene-free benzene until 100 ml. of benzene had been used. The residue that did not dissolve readily in benzene was dissolved in 5 ml. of alcohol and this solution was added to the benzene solution. The benzene solution was shaken thoroughly fifteen times with 100 ml. portions of water. When 500 ml. of aqueous extract had been accumulated, it was washed once with 25 ml. of benzene which were added to the main benzene solution. This procedure was repeated with the second and third 500 ml. accumulations of aqueous extract as they were obtained. The benzene residue was put aside in the refrigerator for further examination. The combined aqueous extracts were concentrated under reduced pressure in a bath kept at 40° to approximately 250 ml. This solution was extracted three times with 50 ml. of chloroform. The extract was filtered and concentrated under reduced pressure to approximately 3 ml. Crystals soon began to separate. After refrigeration for 2 hours the mixture was filtered and the crystals were thoroughly washed with chloroform, which removed almost all of the color. The crystals weighed 187 mg. and melted at 200–205°. A second pool of extracts covering a period of 9 days was treated similarly and yielded 105 mg. of crystals, which melted at 202–205°.

The crude crystals were recrystallized successively from absolute alcohol, acetone, and methanol. The melting point after crystallization from absolute alcohol was 213–215° and was not changed by recrystallization from acetone and then from methanol. The 292 mg. of crude crystals

yielded 191 mg. of recrystallized material. The material that was recrystallized from methanol was prepared for analysis.

$C_{22}H_{32}O_6$. Calculated, C 69.57, H 8.35; found, C 69.81, H 8.49

A few crystals of the isolated material developed a yellow-green fluorescence when treated with a drop of concentrated sulfuric acid. A red dinitrophenylhydrazone precipitated when a small amount of material in 0.5 ml. of alcohol was treated with 0.5 ml. of a solution of 2,4-dinitrophenylhydrazine in 2 N HCl (Brady's reagent).

For determination of the specific rotation 14.7 mg. of substance were dissolved in 5.0 ml. of 95 per cent alcohol: $[\alpha]_D^{26} = +167^\circ \pm 3^\circ$. Reichstein (9) observed $[\alpha]_D^{22} = +167.2^\circ \pm 2^\circ$ ($c = 1.029$ in absolute alcohol).

For examination of the ultraviolet absorption 1.253 mg. were dissolved in 95 per cent alcohol and the solution was made up to 100 ml. Measurements were made with a Beckman spectrophotometer. An absorption maximum was observed at $242 m\mu$; $E_{1cm.} = 0.548$ and $\epsilon = 15,800$ ($\log \epsilon = 4.2$).

Preparation of Acetate—A solution of 24.6 mg. of material in 10 drops of pyridine and 3 drops of acetic anhydride was allowed to stand 24 hours at room temperature. Water and HCl were then added and the precipitate was collected on a filter and washed with water. It was dissolved in hot methanol, the solution was filtered, concentrated to about 1 ml., and cooled. The first crop of crystals melted at $218-219^\circ$. Recrystallization from acetone did not change the melting point. Combination with the second and third crops from the first methanol solution and recrystallization from this solvent gave 19 mg. of large crystals which melted at $219-220^\circ$.

$C_{22}H_{32}O_6$. Calculated, C 68.29, H 7.99; found, C 68.09, H 8.32

Oxidation of 17-Hydroxycorticosterone to Adrenosterone—A solution of 15.0 mg. (0.0414 mm) of the steroid in 1.5 ml. of glacial acetic acid was treated with 0.55 ml. of 0.902 N chromic acid in 90 per cent acetic acid (12×0.0414 milliequivalent). Addition of the chromic acid gave a brown, amorphous precipitate which slowly disappeared on standing. After 20 hours a few drops of methanol were added to destroy any excess chromic acid; then water was added, and the mixture was extracted three times with ethyl acetate. The extract was washed with sodium carbonate solution and water, dried over Na_2SO_4 , and evaporated to dryness. The residue was taken up in a little methanol and dry ether was added. A gelatinous precipitate was centrifuged out and discarded. The solution was evaporated to dryness and the residue was crystallized from dry ether. The first crop weighed 3.6 mg. and melted at $217-219^\circ$. A mixture with

an authentic specimen of adrenosterone (m.p. 218–220°) melted at 217–219°. In 95 per cent alcohol $[\alpha]_D^{26} = +270^\circ \pm 8^\circ$ ($c = 0.1183$). Reichstein (14) observed for adrenosterone (Substance G) $[\alpha]_D = +262^\circ$.

Oxidation of 17-Hydroxycorticosterone Acetate to 17-Hydroxy-11-dehydrocorticosterone Acetate—The acetate (9 mg.; 0.0223 mm) was dissolved in 0.5 ml. of acetic acid, and 0.0669 milliequivalent of chromic acid in 90 per cent acetic acid was added. After 22 hours at room temperature water was added and the mixture was extracted with ethyl acetate. The extract was washed with sodium carbonate solution and water, dried, and evaporated. The residue was crystallized from acetone and gave two crops of 4.0 and 2.2 mg. which melted at 239–240° and 238–240°, respectively. A mixture of the first crop with an authentic specimen of 17-hydroxy-11-dehydrocorticosterone acetate (m.p. 239–240°) melted at 239–240°.

SUMMARY

17-Hydroxycorticosterone has been isolated from the urine in a case of Cushing's syndrome associated with severe diabetes mellitus. From a 25 day collection of urine 191 mg. of purified hormone were obtained.

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FLUOROMETRIC MEASUREMENTS OF RIBOFLAVIN AND ITS NATURAL DERIVATIVES IN SMALL QUANTITIES OF BLOOD SERUM AND CELLS*

By HELEN B. BURCH,[†] OTTO A. BESSEY,[‡] AND OLIVER H. LOWRY,[§]

(From the Division of Nutrition and Physiology, The Public Health Research Institute of The City of New York, Inc., New York)

(Received for publication, March 29, 1948)

The concentrations of many of the vitamins or their natural derivatives in blood serum and cells reflect the relatively recent intake of the particular dietary essential (*e.g.*, ascorbic acid). Such a relationship between blood level and intake has led to the usefulness of chemical analytical methods for the evaluation of nutritional status. However, there is, at present, insufficient information available to indicate whether or not measurements of the blood levels of riboflavin in any of its forms would be helpful in this way. This paucity of data on riboflavin blood levels is primarily attributable to the lack of satisfactory analytical methods. Results with microbiological methods (1-3) have been somewhat discordant, apparently because of the difficulty of measuring the low concentrations of the vitamin in the presence of persistent substances in blood extracts which may enhance or inhibit bacterial growth. The microbiological methods also fail to differentiate between riboflavin and its derivatives. By a manometric technique (4, 5), the flavin-adenine-dinucleotide has been measured in red cells and plasma. This procedure requires so much material and labor as to appear impracticable for wide usage.

The recent development of a very sensitive fluorometer (6) now makes possible methods which are simple, rapid, and reproducible to 3 to 5 per cent, and by which riboflavin can be differentiated from its natural derivatives. With this instrument the fluorescence of as little as 0.2 $\mu\gamma$ of riboflavin in volumes of 0.5 ml. is readily measurable.

Methods will be described for determining (1) both free and total riboflavin in 50 c.mm. of blood serum (25 c.mm. for total alone), (2) the free riboflavin, flavin-mono- and dinucleotide as separate fractions in 0.2 ml. of serum, (3) the total riboflavin in 20 c.mm. of red cells or whole blood, and (4) the total riboflavin in white cells and platelets from 0.1 ml.

* Aided by a grant from the Nutrition Foundation, Inc.

[†] Present address, Department of Chemistry, Columbia University, New York.

[‡] Present address, Department of Biological Chemistry, University of Illinois College of Medicine, Chicago.

[§] Present address, Department of Pharmacology, Washington University School of Medicine, St. Louis.

of blood. Illustrative data include the influence of riboflavin intake on blood levels in the rat, some values for riboflavin concentration in supposedly normal human serum, red blood cells, and white blood cells, and a preliminary study of the effects of large doses and short periods of deprivation of riboflavin on the serum riboflavin of a few human subjects.

EXPERIMENTAL

Principles of Methods—The differentiation of the flavin compounds depends upon the following properties which will be fully discussed elsewhere:¹ (1) At neutrality, on a molar basis, riboflavin and riboflavin monophosphate (flavin-mononucleotide (FMN)) exhibit the same fluorescence, whereas flavin-adenine-dinucleotide (FAD) is only about 14 per cent as fluorescent under the conditions of analysis. (2) Riboflavin is much more readily extracted by benzyl alcohol from aqueous solution than is either FMN or FAD. (3) FAD is completely hydrolyzed to FMN in 5 per cent trichloroacetic acid in 20 hours at 37°.

Materials—

1. Microphotofluorometer fitted with special adapter and tubes as described elsewhere (6) (Farrand Optical Company, Inc., Bronx Boulevard and East 238th Street, New York 66). The commercial instrument requires slightly larger volumes than those used in the present work. The minimum fluid volume required for the particular instrument and cuvettes used should be determined and the corresponding changes made in specimen and reagent volumes.

2. Serological tubes, 6 × 50 mm. (Kimble, No. 45060).

3. Pyrex test-tubes, 3 and 8 ml. capacity.

4. Constriction pipettes 5, 20, 25, 50, 100, 110, 200, 400, and 500 c.mm. (7).

5. Syringe pipette set for 1 ml. volume (Mr. Herman Ruf, 5023 192nd Street, Flushing, Long Island, New York).

6. Pipettes and other equipment described for phosphorus analysis of white blood cells (8).

7. Wide tipped constriction pipette calibrated to contain 20 c.mm. of red blood cells.

8. Trichloroacetic acid, 100 gm. of redistilled acid diluted to 100 ml. From this, 5, 10, and 13 per cent solutions are prepared.

9. 0.16, 2.4, and 4 M K_2HPO_4 solutions.

10. Benzyl alcohol, C.P., redistilled, and saturated with water.

11. Chloroform, C.P., redistilled, and saturated with water.

12. Riboflavin standard solutions, 0.2 to 1.6 γ per ml. in 0.01 N HCl.

¹ Lowry, O. H., Bessey, O. A., and Love, R. H., in preparation.

Although these dilute solutions appear to keep well protected by black cloth in the cold, it is perhaps advisable to prepare them frequently from a stronger stock solution, i.e., 20 γ per ml. in 0.01 N HCl.

13. Sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$). 0.5 gm. dissolved within 30 minutes of use in 5 ml. of 5 per cent sodium bicarbonate. This reagent is preferably kept in a tube in ice water to delay oxidation by the air.

14. Fluorescein secondary standards approximately equivalent in fluorescence to 1, 2, and 4 $m\gamma$ of riboflavin per ml.

15. Reagents as needed for phosphorus determination in white blood cells (8).

16. Potassium oxalate, 1.6 per cent freshly diluted from 8 per cent solution kept in the refrigerator.

17. NaCl, 1 per cent solution.

Because of the small fluorescence measured, reagents must be sufficiently pure, and equipment sufficiently clean so as not to contribute more than a trace of fluorescence to the sample. Glass-redistilled H_2O is used in making all reagents. All tubes are cleaned by boiling first in half concentrated HNO_3 and then, after thorough rinsing, in distilled H_2O .

Since the riboflavin in the neutralized extract is quite sensitive to blue light, it is desirable to work with such extracts in a darkened room equipped with red lamps. Tubes are kept covered whenever possible to prevent contamination.

Some of the reagents listed are not required for certain of the determinations.

Procedure

Determination of Free Riboflavin and Total Riboflavin in Serum—50 c.mm. of serum are well mixed with 1.0 ml. of 5 per cent trichloroacetic acid at 0° in a 3 ml. tube, allowed to stand at $0-5^\circ$ for 15 minutes, and centrifuged in cold cups at $0-5^\circ$ for 10 minutes. 0.4 ml. of the supernatant is transferred to each of two fluorometer tubes, the first of which contains 0.1 ml. of 2.4 M K_2HPO_4 . The remaining sample is reserved for the determination of total riboflavin as described below. The apparent riboflavin content (A) of the tube containing the neutralized extract is measured in the microphotofluorometer within 1 to 2 hours of neutralization. Meanwhile, care is taken to keep the tube in the dark. The tube is carefully wiped with a slightly damp cloth and three readings are made with the same instrument setting against fluorescein: an initial reading, R_1 , a second reading, R_2 , after the addition of an internal riboflavin standard (5 c.mm. equivalent to 1 $m\gamma$ of riboflavin), and a reduced reading, R_3 , after the addition of 5 c.mm. of sodium hydrosulfite solution ("Materials," item (6)). Complete blank determinations are also made on reagents, usually in triplicate. A reading

on a tube containing redistilled water is made as a measure of the contribution from scattered light and possible fluorescence of the tube itself.

For measuring the total riboflavin of the filtrate, the fluorometer tube containing the second sample is stoppered with a clean paraffined cork or very clean dust-free rubber stopper and is allowed to hydrolyze in the dark at 37° for 20 hours, usually overnight. It is then neutralized with 0.1 ml. of 2.4 M K_2HPO_4 and its apparent riboflavin content (B) is measured as above. Great care is taken to mix all solutions thoroughly and to keep the lower portion of the tube free from finger-marks and dust. If carefully calibrated fluorometer tubes are used, it is unnecessary to use an internal standard with more than a few samples, since little or no quenching has been observed with these serum filtrates.

Calculation—The readings R_2 and R_3 are corrected for the dilution resulting from the addition of internal standard and reducing agent, and may be designated R'_2 and R'_3 . (The reading for redistilled water is subtracted from the sample readings before correcting for dilution, since the contribution from scattered light would not be affected by dilution.)

$$\frac{\text{Micrograms riboflavin added}}{\text{Ml. serum in aliquot}} \times \frac{R_1 - R'_2}{R'_2 - R_1} \times 100 = \text{micrograms \% riboflavin}$$

Appropriate correction is made in the above figure for blanks which have been treated and calculated in the same manner as the samples.

Since under the conditions of measurement FAD, before hydrolysis, is 14 per cent as fluorescent as riboflavin, then

$$\text{FAD} = \frac{B - A}{0.86}$$

and

$$\text{Free riboflavin (+FMN)} = A - 0.14\text{FAD} = 1.163A - 0.163B$$

(Ordinarily, very little FMN has been found present in serum.)

If the determination of total riboflavin only is desired, 2.5 c.mm. of serum are sufficient. The serum is precipitated with 0.5 ml. of 5 per cent trichloroacetic acid at room temperature and may be allowed to stand 30 minutes to an hour before centrifuging. A 0.4 ml. aliquot is hydrolyzed and measured as above. Prolonged contact of the protein precipitate with the trichloroacetic acid should be avoided as being likely to increase the blank reading.

Determination of Free Riboflavin, FMN, and FAD—A serum filtrate is prepared at 0–5°, as described above, from 0.2 ml. of serum and 2.0 ml. of 5 per cent trichloroacetic acid in an 8 ml. tube. After centrifuging, 1.0 ml. of the supernatant is transferred into a 3 ml. tube containing 0.25 ml. of 2.4 M K_2HPO_4 and mixed at once. The free riboflavin plus FMN is meas-

ured on a 0.5 ml. aliquot of this sample (*A*). The total riboflavin is measured on a 0.4 ml. aliquot of the remaining supernatant acid extract (*B*) as described above. To 0.75 ml. of the remaining neutral extract, 2 ml. of benzyl alcohol saturated with water are added. Thorough extraction of free riboflavin is accomplished by vigorously agitating the covered tube with a device such as that previously described (9) or by very thorough tapping. After centrifuging 5 minutes at 3000 R.P.M., the benzyl alcohol layer is drawn off as completely as possible by suction. The aqueous layer is then extracted with 1 ml. of chloroform saturated with water and centrifuged. (The chloroform extraction removes the last of the benzyl alcohol and clarifies the aqueous phase.) A 0.5 ml. aliquot of the (upper) aqueous layer is transferred to a fluorometer tube and the apparent riboflavin (*C*) is measured as before.

Calculation—The distribution coefficients for riboflavin, FMN, and FAD between benzyl alcohol and the neutralized trichloroacetic acid extract are 3.8, 0.02, and 0.01 respectively.¹ Thus, when 0.75 ml. of the sample is extracted with 2 ml. of benzyl alcohol, the free riboflavin extracted = $100 \times (3.8 \times 2)/(0.75 + (3.8 \times 2)) = 91$ per cent, leaving 9 per cent in the aqueous phase. Similarly, it may be calculated that 96 per cent FMN and 98 per cent FAD will be left behind after the benzyl alcohol extraction. The FAD left will, as before, show only 14 per cent as much fluorescence as an equivalent amount of riboflavin. Therefore, if *A*, *B*, and *C* are equal to the apparent riboflavin in the three samples above, *A* = free riboflavin + FMN + 0.14FAD, *B* = total riboflavin, and *C* = 0.09 free riboflavin + 0.96FMN + 0.14FAD.

From these equations it may be readily calculated that

$$(1) \quad \text{FAD} = \frac{B - A}{0.86}$$

$$(2) \quad \text{Free riboflavin} = 1.10A - 1.15C + 0.007B$$

For most purposes the extracted FMN is negligible, in which case free riboflavin = $1.10(A - C)$.

$$(3) \quad \text{FMN} = B - \text{FAD} - \text{free riboflavin}$$

Determination of Total Riboflavin in Red and White Blood Cells

Isolation of Blood Cells—The method of isolation of white blood cells and platelets by differential centrifugation from 0.1 ml. of blood has been described (8). The red blood cells which remain after removal of the white cell suspension are sufficient for red cell analysis. The supernatant solution remaining after the white cells are drawn off, together with the upper portion of the red cell layer, is discarded. The remaining red cells are packed by centrifuging at 4° for an hour. Any supernatant liquid is re-

moved and an aliquot of red cells is taken with a dry, rather wide tipped constriction pipette, calibrated to contain 20 c.mm. This is rinsed into 1 ml. of 1 per cent NaCl. The suspension may be stored frozen if immediate analysis is inconvenient.

Analysis of White Blood Cells and Platelets. Riboflavin Measurement—The white blood cells from 0.1 ml. of blood are distributed evenly by vigorous agitation of the small tube and deproteinized by mixing thoroughly with 0.11 ml. of 5 per cent trichloroacetic acid. The sample is allowed to stand at room temperature for 30 minutes to 1 hour and mixed again. After centrifuging at 3000 R.P.M. for 10 minutes, 0.1 ml. of the supernatant fluid is transferred to a fluorometer tube and allowed to hydrolyze at 37°. The residue and remaining supernatant fluid are reserved for phosphorus determination, on which the weight of white cells and platelets is based.

The hydrolyzed filtrate is mixed with 0.4 ml. of 0.16 M K_2HPO_4 and the riboflavin is measured as described for serum. Blanks are provided by treating 0.1 ml. aliquots of 5 per cent trichloroacetic acid in the same manner as the white cell filtrate.

Measurement of Acid-Insoluble Phosphorus—The acid-insoluble phosphorus is measured as previously described (8), except for a simplification made possible because of the more dilute filtrate. Instead of washing the precipitate to remove acid-soluble phosphorus, the 10 c.mm. of acid solution which remain with the precipitate are included in the phosphorus analysis. To reduce the volume of fluid that needs to be evaporated, 20 c.mm. of 7 N H_2SO_4 may be conveniently substituted for the larger volume of 4.5 N acid previously recommended for digestion. Digestion of the sample, color development, and measurement of the absorption at 690 m μ are carried out as described (8).

Calculation—The sample analyzed for phosphorus contains all of the acid-insoluble P and, because of the 10 c.mm. of acid extract not removed, 9 per cent of the acid-soluble P. Since white cells contain an average of 33 micromoles of acid-insoluble P and 28 micromoles of acid-soluble P per gm. (8), the sample contains $33 + 0.09 \times 28 = 35.5$ micromoles of P per gm. of white cells in the sample. Therefore, the micrograms of riboflavin per 35.5 micromoles of P found are numerically equal to the micrograms of riboflavin per gm. of white cells, or

$$(1) \quad \text{Micrograms \% riboflavin} = \frac{\text{micrograms riboflavin in entire sample}}{\text{micromoles P found}} \times 100 \times 35.5$$

Since the riboflavin in the entire sample = riboflavin found $\times 110/100$

$$(2) \quad \text{Micrograms \% riboflavin} = \frac{\text{micrograms riboflavin found}}{\text{micromoles P found}} \times 100 \times 35.5 \times \frac{110}{100}$$

$$(3) \quad \text{Micrograms \% riboflavin} = \frac{\text{millimicrograms riboflavin found}}{\text{micromoles P found}} \times 3.9$$

Analysis of Red Blood Cells—The red blood cell suspension (20 c.mm. in 1 ml. of saline) is well mixed with 3 ml. of 13 per cent trichloroacetic acid and allowed to stand 30 to 60 minutes, mixed again, and centrifuged 10 minutes at 3000 R.P.M. The supernatant liquid, which must be clear, is allowed to hydrolyze at 37°. Aliquots of 0.4 ml. are pipetted into fluorometer tubes containing 0.1 ml. of 4 M K_2HPO_4 . The reducible fluorescence is measured as described above. The reduced readings must be made promptly after the addition of hydrosulfite, as there is a tendency for partial reoxidation of the sample.

Determination of Total Riboflavin in Whole Blood—Total riboflavin may be determined in whole blood in the same manner as in red cells, except that a less dilute extract is prepared. 20 c.mm. of blood are delivered into 2.0 ml. of 10 per cent trichloroacetic acid. (For accuracy the pipette used is calibrated with blood. It is rinsed with dilute ammonia and dried with acetone between samples.) The mixture is shaken promptly to prevent clumping of the precipitate. The rest of the analysis is performed exactly as with red cells. In both cases, it is important that the acid extract does not remain in contact with the protein precipitate for much over an hour at room temperature, or 2 to 3 hours at 4°.

Recovery of Riboflavin and Flavin-Adenine-Dinucleotide—Riboflavin and FAD were added to human whole blood, serum, and red and white blood cells prior to trichloroacetic acid precipitation. Filtrates were prepared, hydrolyzed, and analyzed, at least in triplicate, by the proposed procedures (Table I). Quantitative recovery was obtained. Similar recoveries have been obtained from rat blood, serum, and cells. Recovery, however, was not complete with lower dilutions than those recommended; viz., 1:100 for whole blood, 1:20 for serum, and 1:200 for red cells. In fact, even at 1:200, recovery was found to be incomplete for red cells if 5 per cent trichloroacetic acid was substituted for 10 per cent.

Blood Level and Reproducibility of Analyses—The riboflavin content of serum, white blood cells plus platelets, and red blood cells from small groups of adult human subjects thought to be well nourished is summarized in Tables II and III. The average free riboflavin plus FMN in serum is 0.8 γ per cent, and total riboflavin is 3.2 γ per cent, with ranges of 0.3 to 1.3 and 2.6 to 3.7 γ per cent, respectively. A difference in individuals either as to nutritional level or other factors seems primarily responsible for this rather wide variation, since the methods of analysis are quite reproducible as shown by an estimated standard deviation for the series of 0.07 γ per cent for both the (free + FMN) and FAD. (Estimated standard deviation = $\sqrt{2 \Delta^2 / (2 \times N)}$, where Δ = the difference between duplicate analyses and N = the number of sera analysed. This statistic is equivalent to the true standard deviation that should result from N replicate analyses of one serum.)

In a series of twenty-six duplicate determinations of total riboflavin in serum, the estimated standard deviation was found to be 0.08 γ per cent.

TABLE I
Recovery of Riboflavin and FAD from Human Serum, Whole Blood, and Red and White Blood Cells

Sample	Total riboflavin (initial)	Substance added	Amount added*	Total riboflavin found†	Recovery
	γ per cent		γ per cent	γ per cent	per cent
Serum	3.9	FAD	13.7	17.6	100
"	4.2	Riboflavin	20.3	24.1	98
White blood cells	262	FAD	381	649	101
" " "	262	Riboflavin	546	812	102
Red blood cells	24.3	FAD	94.8	119.4	101
Whole blood	12.2	"	40.6	53.4	102

* Calculated as riboflavin equivalent.

† Average of triplicate analyses.

TABLE II
*Riboflavin Content of Serum from Well Nourished Adults**
The values are recorded as micrograms per cent of riboflavin.

Test subject No.	Free + FMN†	FAD†	Total
1	0.7	3.0	3.7
2	0.9	2.5	3.4
3	1.2	2.5	3.7
4	0.7	2.1	2.8
5	0.9	2.4	3.3
6	0.8	2.1	2.9
7	0.9	2.6	3.5
8	0.5	2.8	3.3
9	0.3	2.4	2.7
10	0.8	1.8	2.6
11	0.4	2.3	2.7
12	1.3	2.2	3.5
13	0.8	1.8	2.6
Average.....	0.8	2.4	3.2
Range.....	0.3-1.3	1.8-3.0	2.6-3.7

* Riboflavin intake thought to be liberal by present nutritional standards.

† Average of duplicate analyses.

The average total riboflavin content of white blood cells plus platelets, and of red blood cells from twelve adults was found to be 252 and 22.4 γ per cent respectively, with ranges of 227 to 293 and 18.0 to 26.2 γ per cent

(Table III). The mean of the twelve standard deviations calculated on three to five samples from each individual was 11 γ per cent for white cells and 1.3 γ per cent for red cells.

Comparison of the analyses of the upper buffy layers of several specimens containing platelets predominantly with the corresponding layers containing leucocytes predominantly has indicated no difference in the riboflavin content of these fractions of the white cell layers.

Stability of Samples—Whole blood samples may be kept as collected at 4° for 48 hours in the dark without measurable change in the concentration

TABLE III
Riboflavin Content of White Blood Cells and Platelets, and of Red Blood Cells from Well Nourished Adults*

The values are recorded as micrograms per cent of riboflavin.

Test subject No.	White blood cells†	Red blood cells†
1	227	18.8
2	243	18.0
3	236	26.2
4	261	21.3
5	245	22.8
6	264	25.0
7	247	25.2
8	293	19.8
9	253	20.8
10	246	21.3
11	242	26.0
12	262	24.3
Average...	252	22.4
Range...	227-293	18.0-26.2

* Riboflavin intake thought to be liberal by present nutritional standards.

† Averages of three to five replicate analyses.

of riboflavin compounds in the serum when it is finally separated. Serum and white and red cells may be stored at -40° for periods of several months without change in the riboflavin content, and, in the case of serum, without change in the proportions of riboflavin, FMN, and FAD.

When FAD is added to serum at room temperature, about 80 per cent of it is hydrolyzed in 1 hour. However, under similar conditions, hydrolysis of the FAD originally present in serum is not detectable after many hours. This would indicate the presence of an enzyme in serum which is capable of splitting uncombined FAD, but which is inactive toward FAD combined with protein. FAD is hydrolyzed in 5 per cent trichloroacetic

acid at the rate of about 5 per cent per hour at 0°, 12 per cent per hour at 15°, and 52 per cent at 38°, which indicates the need for prompt neutralization of extracts to be used for FAD analysis.

Preliminary experiments on the hydrolysis of riboflavin derivatives (FAD) extracted by trichloroacetic acid from serum indicate the possibility of replacing the long acid hydrolysis by enzymatic hydrolysis as an analytical convenience. An enzyme concentrate prepared from potato was used.² Similar rates of hydrolysis were obtained with a purified yeast FAD and with a serum extract. Approximately the same FAD values were obtained

TABLE IV
Riboflavin in Blood Serum, Red Cells, and White Cells of Rats on Various Daily Intakes of Riboflavin

Ribo- flavin in diet	No. of rats	Growth rate	Serum				White blood cells	Red blood cells	Urine
			Free riboflavin	FMN riboflavin	FAD riboflavin	Total riboflavin	Total riboflavin	Total riboflavin	Riboflavin
γ per gm.		gm. per day	γ per cent	γ per cent	γ per cent	γ per cent	γ per cent	γ per cent	γ per mg. creatinine*
0.5	6	1.88	0.14	0.23	1.18	1.55	107	9.6	0.18 (2)
		± 0.22 †	± 0.11	± 0.07	± 0.13	± 0.09	± 7	± 0.4	
1.0	7	2.67	0.28	0.19	1.32	1.78	125	10.8	0.14 (5)
		± 0.14	± 0.07	± 0.05	± 0.05	± 0.05	± 4	± 0.8	
1.5	9	3.53	0.54	0.22	1.36	2.12	142	12.1	0.20 (5)
		± 0.14	± 0.05	± 0.03	± 0.07	± 0.10	± 6	± 0.9	
2.0	7	3.69	0.82	0.38	1.38	2.58	134	12.5	0.16 (5)
		± 0.27	± 0.22	± 0.06	± 0.06	± 0.26	± 3	± 0.8	
3.0	7	4.53	1.59	0.23	1.44	3.27	162	13.6	0.75 (4)
		± 0.35	± 0.08	± 0.05	± 0.09	± 0.18	± 8	± 0.9	
10.0	8	4.29	3.79	0.36	1.66	5.82	164	15.6	13.8 (4).
		± 0.30	± 0.22	± 0.05	± 0.16	± 0.23	± 2	± 0.8	

* The creatinine output was approximately 0.8 mg. per day per rat, or 4.0 mg. per kilo of rat. The figures in parentheses denote the number of rats on which urine analyses were performed.

† Standard error of the mean.

by both acid and enzymatic hydrolysis, which increases confidence in the specificity of the proposed procedure.

Illustrative Data

Relation between Riboflavin Intake and Blood Levels in Rats—The blood of rats receiving diets containing 0.5, 1.0, 1.5, 2.0, 3.0, and 10.0 γ of riboflavin per gm. of diet was analyzed for riboflavin (Table IV). The growth of these rats was improved by successive addition of riboflavin in the diet

² Lowry, O. H., Bessey, O. A., and Love, R. H., in preparation.

up to the 3.0 γ per gm. level. Determinations of free riboflavin, FMN, and FAD were made in duplicate on serum samples. Total riboflavin was measured in white and red blood cells.

The free riboflavin in the serum of rats on the lowest intake level was negligible and increased progressively with each successive increase in the riboflavin of the diet. A marked increase was observed at the highest two levels of intake. In all groups the serum FMN found was very low and was not demonstrably affected by the diet. The FAD in the serum in-

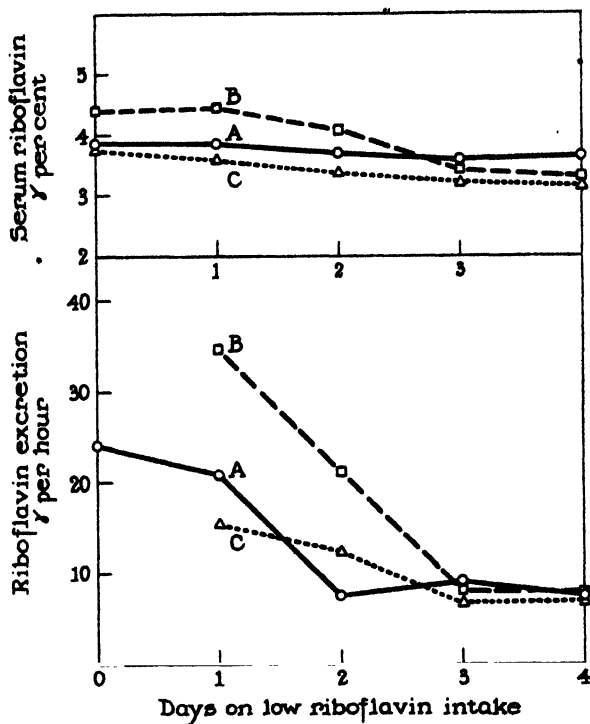


FIG. 1. Serum concentration and urinary excretion of riboflavin of three well nourished human subjects maintained on a low riboflavin diet for 4 days.

creased about 40 per cent from the lowest to the highest intake; *i.e.*, much less than the free riboflavin. Therefore, the changes in the total riboflavin values reflect primarily the changes in the free riboflavin. The group receiving 10.0 γ of riboflavin per gm. of diet had nearly 4 times the total serum riboflavin content of the most deficient group.

The total riboflavin of the white and red cells increased 50 and 60 per cent respectively as the amount of the vitamin in the diet was increased. A comparison of the growth rate in these rats with the blood riboflavin

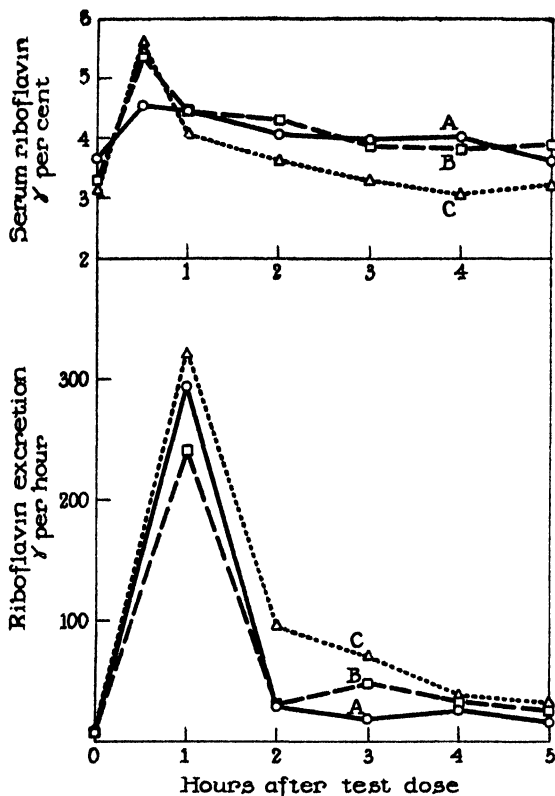


FIG. 2. Serum concentration and urinary excretion of riboflavin of three human subjects following ingestion of 2 mg. of riboflavin.

values (Table IV) suggests possible diagnostic value for the riboflavin measurements. The relation between the serum values and the urinary excretion is of interest.³

³ The rat urine was analyzed as follows: 50 c.mm. of urine buffered with 5 c.mm. of 3.25 M sodium acetate-acetic acid (pH 4.6) were oxidized with 10 c.mm. of 4 per cent KMnO_4 for 1 minute. Oxidation was stopped by the addition of 2 c.mm. of 30 per cent H_2O_2 . 50 mg. of $(\text{NH}_4)_2\text{SO}_4$ (previously washed with 95 per cent alcohol and ether to remove fluorescent substances) and 40 c.mm. of benzyl alcohol saturated with water were added. Extraction of the riboflavin was accomplished by vigorous shaking of the tube (9). After centrifuging, 20 c.mm. of the benzyl alcohol layer were pipetted into 0.5 ml. of 45 per cent ethyl alcohol which was 0.1 N in acetic acid and 0.1 N in sodium acetate. The riboflavin was measured as reducible fluorescence compared with an appropriate internal standard in the microfluorometer. The urine from human subjects was similarly analyzed on a macro scale with 4 ml. of urine plus 0.4 ml. of 3.25 M buffer, 1 ml. of KMnO_4 , 0.1 ml. of H_2O_2 , 4 gm. of $(\text{NH}_4)_2\text{SO}_4$, and 3 ml. of benzyl alcohol, of which 1 or 2 ml. were pipetted into 7 ml.

Comparison of Serum and Urinary Levels of Riboflavin in Man—Three human subjects restricted their riboflavin intake for 4 days to a level of about 0.4 mg. per day. The total riboflavin in the serum dropped only 8, 16, and 25 per cent from the initial levels for the three subjects, whereas the urinary excretion (5 hour collection period) decreased to a fourth to a half of the initial rate³ (Fig. 1).

The morning of the 5th day, each person received 2 mg. of riboflavin with 600 ml. of water and a few crackers. Serum samples were collected from the finger after 30 minutes and both serum and urine samples were taken at hourly intervals thereafter. The greatest increase in serum riboflavin occurred at 30 minutes (24 to 75 per cent, Fig. 2). An abrupt decrease had already occurred within an hour following the large dose. Thereafter, the serum levels promptly returned to approximately initial values. The increase in urinary riboflavin output was relatively much greater (30- to 45-fold during the 1st hour) and more prolonged than the changes in the serum concentrations. This greater stability of the serum riboflavin would, however, be of diagnostic value only if the levels in the serum reflect the nutritional status as they seem to in the rat. Unpublished data on a few deficient subjects studied with Dr. L. Emmett Holt, Jr., suggest that the total riboflavin in man may be too stable to be of value, but that the free serum riboflavin may prove useful as a measure of nutritional status.

SUMMARY

Methods are described for the measurement of (1) both free and total riboflavin in 50 c.mm. of blood serum (25 c.mm. for total alone), (2) free riboflavin and flavin-mono- and dinucleotide as separate fractions in 0.2 ml. of serum, (3) total riboflavin in 20 c.mm. of red cells or whole blood, and (4) total riboflavin in white cells and platelets from 0.1 ml. of blood. Determinations show a standard deviation of 0.08 γ per cent on serum, 11 γ per cent on white cells, and 1.3 γ per cent on red cells. Two analysts can perform 75 to 100 riboflavin analyses on serum in 2 days, or twenty-five to 50 on red and white cells.

In thirteen adult human sera, from presumably adequately nourished subjects, the average free riboflavin plus FMN was 0.8 and the FAD 2.4 γ per cent; in the white cells and platelets and in the red cells of twelve of these subjects, the average riboflavin content was 252 and 22.4 γ per cent respectively.

Data for the rat are reported on the relationship between the riboflavin in the diet and the riboflavin of the serum, red cells, white cells, and urine.

of the 45 per cent alcohol buffer for measurement of the reducible fluorescence. The oxidation and extraction were designed to avoid interference from reducible fluorescent substances which are oxidised or which are more water-soluble than riboflavin.

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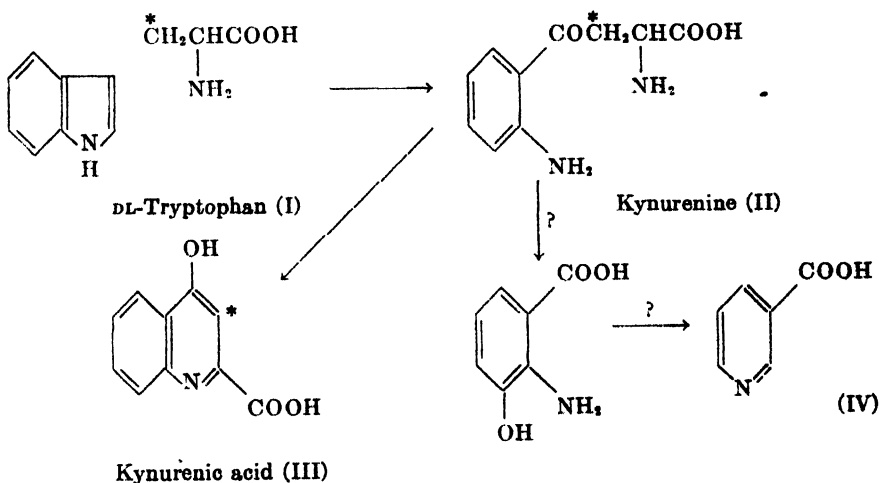
LETTERS TO THE EDITORS

CONCERNING THE MECHANISM OF THE MAMMALIAN CONVERSION OF TRYPTOPHAN INTO KYNURENINE, KYNURENIC ACID, AND NICOTINIC ACID*

Sirs:

The metabolic conversion of tryptophan (I) into kynurenine (II),¹ kynurenic acid (III),² and nicotinic acid (IV)³ (largely excreted as the amide of the N-methyl derivative⁴) has been demonstrated by feeding experiments in a number of mammals. That kynurenine and 3-hydroxyanthranilic acid are intermediates in the latter conversion has been demonstrated in *Neurospora*.⁵

We have synthesized⁶ DL-tryptophan- β -C¹⁴ and are studying the mechanism of these conversions:



* This paper is based on work performed under contract No. W-7405-eng-48 with the Atomic Energy Commission in connection with the Radiation Laboratory, University of California, Berkeley.

¹ Butenandt, A., Weidel, W., Weichert, R., and von Derjugin, W., *Z. physiol. Chem.*, **279**, 27 (1943).

² Ellinger, A., *Z. physiol. Chem.*, **43**, 325 (1904).

³ Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **150**, 395 (1943).

⁴ Hundley, J. M., and Bond, H. W., *J. Biol. Chem.*, **173**, 513 (1948).

⁵ Beadle, G. W., Mitchell, H. K., and Nyc, J. F., *Proc. Nat. Acad. Sc.*, **33**, 155 (1947).

⁶ Heidelberger, C., unpublished material.

The tryptophan was administered to rabbits, dogs, and rats, and from the urine kynurenine, kynurenic acid, and N-methylnicotinamide, respectively, were isolated by the usual methods.

The position of the label in kynurenine was proved by its conversion with alkaline hypoiodite to iodoform of correct specific activity. The position of the labeled carbon atom in the kynurenic acid was indicated by another series of reactions, which shows that these conversions occur as formulated above.

After the carrier N-methylnicotinamide was added to the permutit eluate of rat urine already containing 115 γ (determined fluorometrically), it was isolated as the picrate, and was completely devoid of radioactivity. This proves, on the basis of the specific activities employed, that less than 0.005 per cent of the nicotinic acid produced from the tryptophan could have arisen by oxidation of a molecule such as kynurenic acid which already contains a pyridine ring, because such a mechanism would result in a tagged nicotinic acid.

*Department of Chemistry and the
Radiation Laboratory
University of California
Department of Home Economics
and the Division of Poultry Husbandry
Agricultural Experiment Station
University of California
Berkeley*

CHARLES HEIDELBERGER
MARY E. GULLBERG
AGNES FAY MORGAN
SAMUEL LEPKOVSKY

Received for publication, June 1, 1948

THYMINE DESOXYRIBOSIDE AS AN ESSENTIAL GROWTH FACTOR FOR LACTIC ACID BACTERIA*

Sirs:

Recent findings of Shive *et al.*¹ that thymidine (or a closely related compound) was highly active in counteracting growth inhibition of *Leuconostoc mesenteroides* 8293 by methylfolic acid in a medium which contained thymine suggested that conversion of thymine to thymidine might not occur

Essential Nature of Thymine Desoxyriboside for Certain Lactic-Acid Bacteria

Additions to basal medium*		Turbidity†		
		<i>Lactobacillus leichmannii</i> 313	<i>Lactobacillus leichmannii</i> 327	<i>Leuconostoc citroverum</i>
	γ per 10 cc.			
Thymine desoxyriboside	0	99	99	89
	2	90	86	72
	4	81	74	58
	10	57	57	41
	20	54	47	37
	100	47	47	37
Thymine	100	99	99	84

* The medium contained, per 10 cc., glucose 100 mg., sodium acetate 100 mg., sodium citrate 100 mg., enzymatic casein digest 6 mg., acid-hydrolyzed casein 50 mg., K_2HPO_4 30 mg., KH_2PO_4 30 mg., $MgSO_4 \cdot 7H_2O$ 28 mg., $MnSO_4 \cdot H_2O$ 6 mg., $FeSO_4 \cdot 7H_2O$ 1.7 mg., Tween 80 10 mg., oleic acid 0.1 mg., asparagine 1 mg., DL-tryptophan 1.0 mg., L-cystine 2 mg., *p*-aminobenzoic acid 1 γ , biotin 0.02 γ , pteroyl-glutamic acid 0.1 γ , niacin 10 γ , calcium pantothenate, pyridoxal, pyridoxamine, and thiamine 2 γ each, riboflavin 4 γ , adenine, guanine, and uracil 100 γ each. Acid-hydrolyzed and enzymatically digested caseins were charcoal-treated preparations.

† Per cent of incident light transmitted, uninoculated medium = 100; incubation time, 20 to 24 hours at 37°. No growth without thymidine occurred, even though incubation was continued for 3 days.

in certain lactic acid bacteria, and that thymidine might therefore be essential for growth of some of these organisms in the absence of growth inhibitors.

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the United States Public Health Service. We are indebted to Dr. William Shive and Dr. Walter C. Schneider for samples of thymine desoxyriboside, and to Dr. Shive for information about his experiments in advance of their publication.

¹ Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Am. Chem. Soc.*, **70**, 2299 (1948).

This compound was consequently tested for its ability to replace unidentified natural extracts required for growth of several lactic acid bacteria in media of known composition. From the table it is apparent that thymine desoxyriboside is an essential growth factor for several organisms of this group. Similar data show that it also permits growth of several strains of *Lactobacillus acidophilus* in a similar medium. Thymine is without activity for each of these organisms, and the medium contains an excess of folic acid.

These data indicate a fairly wide-spread requirement for this compound among that group of lactic acid bacteria which have previously failed to grow in media of known composition.

Department of Biochemistry
University of Wisconsin
Madison

ESMOND E. SNELL
ESTELLE KITAY
WALTER S. McNUTT

Received for publication, June 5, 1948

THE ABILITY OF THYMIDINE TO REPLACE VITAMIN B₁₂ AS A GROWTH FACTOR FOR CERTAIN LACTOBACILLI

Sirs:

Shive *et al.*¹ recently have reported the isolation from liver of a crystalline factor functionally related to folic acid. Preliminary investigation of the structure by Dr. Shive indicates that the compound is thymidine (thymine desoxyriboside). Dr. Shive has kindly furnished us with a sample of the

Experiment No.	Material per tube assayed		Turbidity
1	None	γ	0
	Liver concentrate	80	4
		160	20
		240	41
		400	104
	Thymidine	0.4	15
		0.8	43
		1.2	106
2		2.0	130
	None		0
	Liver concentrate	16	29
		32	60
		64	98
		160	137
	Thymine	1	4
		2	1
		3	4
		5	6
		100	10
		200	0
		300	1
		500	5

The medium was that of Wright and Skeggs³ modified by the addition of 1 γ of biotin, 100 γ of folic acid, 500 mg. of norit-treated tryptic digest of casein, 200 mg. of Tween 80, and 10 ml. of clarified tomato juice per 100 ml. of double strength medium.

isolated factor for microbiological study. We have found that for certain lactic acid bacteria thymidine is able to replace the requirement for vitamin B₁₂.² The data obtained with *Lactobacillus lactis* (ATCC 8000) are being reported together with our interpretation of these findings.

¹ Shive, W., Eakin, R. E., Harding, W. M., Ravel, J. M., and Sutherland, J. E., *J. Am. Chem. Soc.*, **70**, 2299 (1948).

² Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, **107**, 396 (1948).

³ Wright, L. D., and Skeggs, H. R., *Proc. Soc. Exp. Biol. and Med.*, **56**, 95 (1944).

The table demonstrates that growth comparable to that obtained with liver is observed in the presence of 0.4 to 2.0 γ of thymidine per tube. Thymine is inactive under these conditions. Obviously, from the comparatively large amounts of thymidine required for optimal growth of the organism, thymidine is not vitamin B₁₂. We interpret these data as indicating that vitamin B₁₂ functions as a coenzyme in carrying out reactions concerned with conversion of thymine to thymidine, since in the presence of thymidine the vitamin no longer is required by *Lactobacillus lactis*.

By analogy the microbiological evidence reported in this paper indicates that the primary biochemical defect in pernicious anemia may well be inability to synthesize certain nucleosides, particularly thymidine, from parent purines or pyrimidines. Thus it would appear that the curative effects observed in this disease with folic acid arise from increased thymine synthesis,⁴ which by mass action effects yields more thymidine. The effectiveness of large amounts of thymine in pernicious anemia⁵ similarly may be explained.

Medical Research Division
Sharp and Dohme, Inc.
Glenolden, Pennsylvania

LEMUEL D. WRIGHT
HELEN R. SKEGGS
JESSE W. HUFF

Received for publication, June 7, 1948

⁴ Stokes, J. L., *J. Bact.*, **47**, 27 (1944).

⁵ Spies, T. D., Vilter, C. F., Cline, J. K., and Frommeyer, W. B., *Southern Med. J.*, **39**, 269 (1946).

MYOSIN AND ADENYLPYROPHOSPHATASE IN INSECT MUSCLE*

Sirs:

In the course of an investigation on the adenylypyrophosphatase of insect muscle, preparations of myosin (thrice precipitated by the method of Bailey¹) were found to have a very low activity (0 to 360 γ of phosphorus split off per mg. of protein nitrogen in 5 minutes at 37°). An examination of the steps of the method revealed that a large part of the adenylypyrophosphatase activity had been washed out of the myosin at the first precipitation.

Enzyme preparation	Experiment No.	Total protein N	Activity
		mg.	γ Per mg. N per 5 min.
Myosin (once pptd.)	1	73.5	280
	2	54.0	360
Supernatant from 1st pptn. (20 volumes)	1	38.9	490
	2	34.1	710
Myosin (thrice pptd.)	1	13.8	280
	2	13.3	230

In the method used, the thoraces and hind femora of grasshoppers were ground with 5 volumes of a salt solution containing KCl (0.5 M) and NaHCO₃ (0.03 N), and extracted for 1 hour in the cold. The insoluble material was spun off, and the myosin precipitated from the extract by diluting with 20 to 30 volumes of cold water of pH 7.0. After standing, the myosin was centrifuged down and dissolved in 0.05 M veronal-HCl buffer, pH 8.0, containing 0.5 M KCl. Enzyme activity was tested against either rabbit or grasshopper ATP at pH 8.0, in the presence of excess substrate and 0.001 M MgCl₂.

The distribution of adenylypyrophosphatase activity between the myosin and the supernatant solution is shown in the table. About 50 per cent of the total apyrase was washed from the myosin and remained in aqueous solution, the activity of the enzyme per mg. of nitrogen being consistently higher in the supernatant solution. Repeated precipitation of the myosin

* The work described in this letter was carried out as part of the research program of the Division of Economic Entomology of the Council for Scientific and Industrial Research, Australia.

¹ Bailey, K., *Biochem. J.*, **36**, 121 (1942).

reduced the residual adenylypyrophosphatase activity only slightly (see the table); in any experiment where there was a very marked reduction (*e.g.*, after four precipitations) the possibility of inactivation of the enzyme could not be discounted.

Similar results were obtained when myosin was precipitated by dialysis against 0.005 M veronal buffer (method of Singher and Meister²). By using this method it was possible to obtain concentrated aqueous solutions of the adenylypyrophosphatase.

The lack of a firm union between adenylypyrophosphatase and myosin was confirmed by testing simple water extracts of muscle. It was found that more than half of the total apyrase activity was present in the water extract, as compared with less than one-tenth of the total in rabbit muscle (Needham³).

Further work on the purification of the adenylypyrophosphatase is in progress. Preparations so far obtained split both labile phosphates from the ATP molecule, and no evidence has been found that more than 1 enzyme is involved. Grasshopper muscle also contains an enzyme which hydrolyzes sodium pyrophosphate. This may be separated from the adenylypyrophosphatase by ammonium sulfate fractionation.

*Council for Scientific and
Industrial Research
Canberra, Australia*

D. GILMOUR

Received for publication, June 11, 1948

² Singher, H. O., and Meister, A., *J. Biol. Chem.*, **159**, 491 (1945).

³ Needham, D. M., *Biochem. J.*, **36**, 113 (1942).

THE EFFECT OF 3-HYDROXYANTHRANILIC ACID ON THE EXCRETION OF NIACIN BY THE RAT

Sirs:

Recent work by Mitchell and Nyc¹ on the synthesis of niacin by the mold *Neurospora* shows that this synthesis proceeds from tryptophan through kynurenine and 3-hydroxyanthranilic acid as intermediates. The rôle of tryptophan as a precursor of niacin in rats has been considered, for example, by Krehl *et al.*,² but Ellinger and Abdel Kader³ concluded that this was a very unlikely pathway on chemical grounds. Rosen *et al.*⁴ were unable to demonstrate an increase in end-products of niacin metabolism

Diet	Average excretion, γ per rat per day			
	Apr. 20-23		Apr. 23-26	
	Niacin	N ¹⁵ -Methyl-nicotinamide	Niacin	N ¹⁵ -Methyl-nicotinamide
Basal*	2.6	4.0	5.8	4.1
" + 0.02 mg. niacin per 100 gm.	12.0	44.4	14.0	34.9
" + 1 mg. tryptophan per 100 gm.	10.0	31.5	12.0	39.6
" + 0.1 mg. 3-hydroxyanthranilic per 100 gm.	2.9	2.0	4.0	7.5
" + 1 mg. 3-hydroxyanthranilic per 100 gm.	16.0	62.5	17.0	56.6

* The basal diet consisted of approximately 91 per cent white corn-meal and 9 per cent casein. The niacin content of the basal diet was 450 γ per 100 gm., as shown by microbiological assay.

after feeding kynurenine to rats, and Bonner⁵ reported preliminary studies indicating that 3-hydroxyanthranilic acid would not replace niacin in rat growth.

Mitchell and coworkers⁶ have, however, concluded experiments demonstrating that the growth of rats on a commercial casein diet is greater when the diet is supplemented with 3-hydroxyanthranilic acid (1 mg. per 100 gm. of diet) than when supplemented with tryptophan at the same level.

¹ Mitchell, H. K., and Nyc, J. F., *Proc. Nat. Acad. Sc.*, **34**, 1 (1948).

² Krehl, W. A., de la Hueraga, J., and Elvehjem, C. A., *J. Biol. Chem.*, **164**, 551 (1946).

³ Ellinger, P., and Abdel Kader, M. M., *Nature*, **160**, 675 (1947).

⁴ Rosen, F., Huff, J. W., and Perlzweig, W. A., *J. Nutr.*, **33**, 561 (1947).

⁵ Bonner, D., *Proc. Nat. Acad. Sc.*, **34**, 5 (1948).

⁶ Mitchell, H. K., Nyc, J. F., and Owen, R. D., *J. Biol. Chem.*, in press (1948).

Small amounts of hydroxyanthranilic acid, however, produced no growth response.

The rats used for the growth experiments of Mitchell *et al.*⁶ were made available to us for metabolism studies.⁷ The rats were continued on the experimental diets *ad libitum*, and urine was collected for two 72 hour periods. Urine samples from four rats in each group were pooled for analysis. Niacin was determined by microbiological assay (Hawk, Oser, and Summerson⁸) without hydrolysis, and N¹-methylnicotinamide by the fluorometric method of Huff and Perlzweig.⁹ The results are presented in the table.

These results, taken in conjunction with the growth studies of Mitchell *et al.*,⁶ indicate that 3-hydroxyanthranilic acid is capable of replacing tryptophan in the biosynthesis of niacin in the rat. In view of the fact that tryptophan, kynurenine, and 3-hydroxyanthranilic acid will support growth in various mutant strains of *Neurospora*, that tryptophan and 3-hydroxyanthranilic acid will replace niacin for growth in the rat, and that administration of tryptophan or 3-hydroxyanthranilic acid results in an increase in the excretion of niacin and its metabolites, there appears to be no reason to doubt that the synthesis of niacin in the rat proceeds from tryptophan through 3-hydroxyanthranilic acid as an intermediate.

Department of Biochemistry and Nutrition
University of Southern California
School of Medicine
Los Angeles

PAUL W. ALBERT
BRADLEY T. SCHEER
HARRY J. DEUEL, JR.

Received for publication, June 21, 1948

⁷ We are indebted to Dr. Mitchell, Dr. Nyc, and Dr. Owen of the California Institute of Technology for supplying the diets and groups of rats, immediately following the completion of their growth experiments.

⁸ Hawk, P. B., Oser, B. L., and Summerson, W. H., *Practical physiological chemistry*, Philadelphia, 12th edition, 1095-1097 (1940).

⁹ Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **167**, 157 (1947).

A NEUROSPORA MUTANT DEFICIENT IN THE ENZYMATIC SYNTHESIS OF TRYPTOPHAN*

Sirs:

Until recently there has been no demonstrated case in *Neurospora* in which the loss of activity of an essential enzyme could be attributed to gene mutation. It has been assumed from indirect evidence¹ that gene mutations do cause such losses and thereby result in "blocks" of essential biochemical reactions. The products of the "blocked" reactions thus become essential growth substances for the mutants. The first success in obtaining direct evidence to support the above assumption has been attained by Wagner and Guirard² in studies on the enzymatic synthesis of pantothenic acid by cell-free extracts of *Neurospora*.

Enzymatic Synthesis of Tryptophan with 5 Ml. of Crude Enzyme Containing 2.34 Mg. of Indole, 5.0 Mg. of DL-Serine, and 0.1 Mg. of Pyridoxal Phosphate
Total volume, 10 ml.; 5 hours reaction at 37°.

Experiment No.	Strain used for enzyme preparation	Tryptophan produced	Indole converted
		mg.	per cent
1	Wild type 6A	1.30	32
	C-83	0.00	0
	10,575*	0.99	24
2	Wild type 6A	2.50	62
	C-83	0.00	0
	39,410*	2.15	53

* Tryptophan-requiring strains of a different genetic constitution from that of strain C-83.

Recently, as a result of the development of a method for selecting specific mutants,³ a second example of a loss of enzyme activity as the result of a gene mutation has been discovered. A mutant was obtained that required tryptophan for growth but could not utilize indole. Cell-free extracts of this mutant (strain C-83) lack the ability to couple indole and serine to give tryptophan. As reported by Umbreit, Wood, and Gunsalus,⁴

* This work was supported in part by a grant from the Rockefeller Foundation, and in part under contract with the Office of Naval Research, United States Navy Department.

¹ Beadle, G. W., *Physiol. Rev.*, **25**, 643 (1946).

² Private communication; in press.

³ Lein, J., Mitchell, H. K., and Houlahan, M. B., *Proc. Nat. Acad. Sc.*, in press (1948).

⁴ Umbreit, W. W., Wood, W. A., and Gunsalus, I. C., *J. Biol. Chem.*, **165**, 731 (1946).

the enzyme system capable of carrying out this reaction can be extracted from wild type *Neurospora*. Pyridoxal phosphate is required as a co-enzyme.

In the present work, crude enzyme preparations have been made as described by the previous investigators.⁴ The yield of tryptophan from the enzymatic reaction was determined by using mutant C-83 as an assay organism. This strain requires tryptophan for growth and is neither stimulated nor inhibited by indole, anthranilic acid, kynurenine, 3-hydroxy-anthranilic acid, or nicotinic acid.⁵ Some of the data that have been obtained are summarized in the table.

Although not included in the table, controls were carried out to show that the crude enzyme preparations did not contain an assayable quantity of tryptophan unless they were incubated in the presence of indole and serine. As shown in the table, a preparation from strain C-83 contained no tryptophan even when incubated in the presence of indole, serine, and pyridoxal phosphate.

We are indebted to Dr. I. C. Gunsalus for the pyridoxal phosphate used.

*Kerckhoff Laboratories of Biology
California Institute of Technology
Pasadena*

HERSCHEL K. MITCHELL
JOSEPH LEIN*

Received for publication, June 23, 1948

* Mitchell, H. K., and Nyc, J. F., *Proc. Nat. Acad. Sc.*, **34**, 1 (1948).

* Merck Fellow.

EVIDENCE FOR THE NATURAL OCCURRENCE OF α -AMINO- β , β -DIMETHYL- γ -HYDROXYBUTYRIC ACID (PANTONINE)

Sirs:

Pantoinine, α -amino- β , β -dimethyl- γ -hydroxybutyric acid, has been proposed as a metabolic intermediate in the biosynthesis of pantoic acid. It has been demonstrated that this amino acid is biologically active in nullifying the toxic effect of salicylic acid on *Escherichia coli*.¹

In studies with the technique of paper chromatography, it was found that in several solvents the migration of synthetic pantonine closely followed that of α -aminobutyric acid. Since Polson,² using the same technique, reported an unknown amino acid in acid-hydrolyzed *Escherichia coli* cells with migration characteristics also similar to α -aminobutyric acid, an investigation was made to determine whether this hydrolysate would serve as a natural source of pantonine.

Amino acids	<i>R_F</i> values in solvents			
	Isobutyric acid	Phenol	2, 6-Lutidine	2, 4, 6-Collidine
Unknown.	63	69	48	43
DL-Pantoinine	63	69	48	43
DL- α -Aminobutyric	61	64	38	39
DL- α -Aminoisobutyric	59	68	40	37

The capillary ascent modification³ of the paper chromatographic technique was used and the positions of the amino acids were indicated by developing a color with ninhydrin. The table contains the *R_F* values obtained for certain amino acids, including the unknown one with a number of solvents. The hydrolysate was prepared by allowing the bacterial cells to stand 4 days in 6 N hydrochloric acid at room temperature. This hydrolysate was brought to dryness on a steam cone and freed of chloride ions with silver carbonate. The concentrate was paper-chromatographed in isobutyric acid and that section of the chromatogram corresponding to pantonine was eluted with water to supply the material used in determining the migration of the unknown in collidine, lutidine, and phenol.

¹ Ackermann, W. W., and Shive, W., *J. Biol. Chem.*, in press.

² Polson, A., *Nature*, **161**, 351 (1948).

³ Williams, R. J., and Kirby, H., *Science*, **107**, 481 (1948).

From the table it will be observed that, in 2,6-lutidine, α -aminobutyric acid and α -aminoisobutyric acid migrate more slowly than synthetic pantonine or the unknown material. The R_f values obtained for the unknown in isobutyric acid and phenol correspond to no known naturally occurring amino acid nor to norvaline or norleucine. In the four solvents tested, the R_f values of the unknown and synthetic pantonine are in agreement.

These data indicate that acid-hydrolyzed *Escherichia coli* cells contain, as previously reported, at least one unknown amino acid; this acid is not α -aminobutyric acid but, in four solvents tested, behaves like pantonine.

As a result of these data an effort to isolate this material from acid hydrolysates of *Escherichia coli* cells has been undertaken with paper chromatography as an assay method.

*Biochemical Institute of The University of Texas
and the Clayton Foundation for Research
Austin*

W. W. ACKERMANN
HELEN KIRBY

Received for publication, July 8, 1948

THE NATURE OF BACITRACIN

Sirs:

It has proved feasible to study bacitracin by counter-current distribution in *sec*-butanol-1.7 per cent aqueous acetic acid. Material from a commercial source,¹ assaying 46 u per mg., gave a major band (83 per cent of sample) and two smaller bands. Bioassay² of the fractions gave a single band superimposable on the major band shown by weight analysis. Hydrolysis of the major band and subsequent paper chromatography revealed nine amino acids.

The material in the major band could be recovered without loss of activity by lyophilization. A hygroscopic white powder (activity = 57 u per mg.) which had little or no optical activity was thereby obtained. Redistribution of this material gave only a single band. The polypeptide nature of the active principle is thus strongly indicated.

In an experiment designed to permit isolation of some of the individual acids, 3 gm. of the crude material were hydrolyzed with 6 M HCl at 100° for 22.5 hours, evaporated, and the remaining halogen removed with silver acetate and the silver with H₂S. After evaporation the mixed free amino acids were studied by counter-current distribution in a 54 tube apparatus with the system *sec*-butanol-30 per cent aqueous ammonium acetate containing about 7 per cent ammonia. With the single withdrawal technique³ and 300 transfers, five bands appeared in the withdrawn section of the curve. The first band emerging was a crystalline dipeptide with little or no optical activity, but which contained phenylalanine and isoleucine.

C₁₅H₂₅O₄N₂. Calculated. C 64.72, H 7.96, N 10.06

Found. " 64.37, " 8.08, " 9.89

Van Slyke NH₂, calculated, 5.04; found, 5.05

The next band was a peptide which apparently contained phenylalanine and ornithine. The third band proved to be racemic phenylalanine. Leucine, $[\alpha]_D^{25} = -6^\circ$ ($C = 1.0$ in H₂O), $+14^\circ$ ($C = 0.66$ in 13 per cent HCl) was isolated from the fourth band. Isoleucine, $[\alpha]_D^{25} = +4^\circ$ ($C = 1.35$ in H₂O), $+18^\circ$ ($C = 0.9$ in 13 per cent HCl) was isolated from the fifth band. The material not as yet withdrawn from the apparatus contained mixtures of the other amino acids.

It would appear that at least part of the amino acids of bacitracin are

¹ Sample kindly supplied by the Commercial Solvents Corporation, Terre Haute, Indiana.

² We are indebted to Dr. R. E. Bennett and Mr. G. H. Craig of the Commercial Solvents Corporation for the bioassays.

³ Craig, L. C., *J. Biol. Chem.*, **155**, 519 (1944).

of the unnatural configuration, but the possibility of partial racemization during hydrolysis cannot be excluded.

Preliminary amino acid analyses by the starch column technique⁴ have been kindly carried out by Dr. Moore and Dr. Stein on purified material. After refluxing for 18 hours with 6 M HCl, there was evidence for the presence of a few per cent of the material still in peptide form. The following approximate composition in terms of gm. of amino acid per 100 gm. of bacitracin was found; phenylalanine 11, leucine 9, isoleucine 22, glutamic acid 10, aspartic acid 17, lysine 9, histidine 10, cystine (from the total sulfur) 14, and ammonia 1.5. An additional component was found to move on the column at the same rate as cystine. Two amino acids known to move at this rate are ornithine and hydroxylysine. The chromatograms have demonstrated that, within the sensitivity of the method (0.1 per cent), the following amino acids are absent; methionine, valine, threonine, serine, proline, and arginine.

*The Rockefeller Institute
for Medical Research
New York*

GUY T. BARRY
J. DELAFIELD GREGORY
LYMAN C. CRAIG

Received for publication, July 3, 1948

⁴ Stein, W. H., and Moore, S., *J. Biol. Chem.*, in press. Moore, S., and Stein, W. H., *Ann. New York Acad. Sc.*, **49**, 265 (1948). Stein, W. H., and Moore, S., *Federation Proc.*, **7**, 192 (1948).

REVERSIBLE INACTIVATION OF ALKALINE KIDNEY PHOSPHATASE

Sirs:

Albers *et al.*¹ postulated the existence of a prosthetic group of phosphatases and supplied experimental data to show that the dissociation of the holoenzyme into coenzyme and apoenzyme was dependent on the pH of its solutions. Albers² inactivated alkaline kidney phosphatase by dialysis and observed partial reactivation on addition of dialysate to the incubation mixture. He concluded that phosphatase contains a dialyzable prosthetic group. Von Euler and Hahn³ interpreted the partial reactivation of intestinal phosphatase (inactivated by dialysis at pH 4.5) by the addition of boiled enzyme as proof for the existence of a dialyzable organic cophosphatase. Roche *et al.*⁴ found that incubation with amino acids in alkaline solution reactivated dialyzed intestinal phosphatase.

Experiments carried out in this laboratory revealed that mere incubation with alkaline buffer (pH 9.5) was considerably more effective in reactivating dialyzed alkaline kidney phosphatase than was the addition of dialysate. Furthermore, no significant difference in the rate of inactivation was observed, whether phosphatase in slightly acid solution was dialyzed or merely stored at 4°. The effectiveness of dialysate to restore activity decreased during the course of dialysis. This was not due to a chemical change of the dialysate but to a diminishing response of the protein, as was seen by comparing the effect of fresh and aged dialysates on phosphatases at various stages of dialysis.

Our observations do not support the theory of the existence of a dialyzable cophosphatase. Dialysis probably removed activators and not a prosthetic group. Denaturation of protein may be mainly responsible for the inactivation of alkaline kidney phosphatase during dialysis. A partial

¹ Albers, H., Beyer, E., Bohnenkamp, A., and Müller, G., *Ber. chem. ges.*, **71**, 1913 (1938).

² Albers, D., *Z. physiol. Chem.*, **261**, 43, 269 (1939).

³ von Euler, H.; and Hahn, L., *Experientia*, **3**, 412 (1947).

⁴ Roche, J., van Thoai, N., and Roger, M., *Compt. rend. Soc. biol.*, **138**, 653 (1944).

reversal of this denaturation by incubation in alkaline solution would explain the restoration of activity without addition of dialysate.

Experiment No	Hrs. dialyzed	Hrs. stored	Temperature	pH of dialysis	Mg. P liberated per hr. per mg. enzyme		
					No addition	Dialysate added	Incubated 2 hrs., pH 9.5; no addition
			°C				
8-C		24	37	7.0	9.12		
8-D	24		37	7.0	6.99	7.80	8.63
5-C		72	37	7.0	8.54		
5-D	72		37	7.0	1.51	1.63	4.26
3-C		137	4	7.0	9.06		
3-D	137		4	7.0	5.59	6.34	8.22
18-CN		71	4	7.0	8.69		
18-C5		71	4	5.4	0.95		5.90
18-D5	71		4	5.4	0.78	0.96	4.97
18-C4		142	4	4.2	0.25		3.38
18-D4	71	71	4	4.2	0.26		3.50
21-C		71	4	9.5	8.70		7.88
21-D	71		4	9.5	8.32	8.16	7.88

Purified alkaline kidney phosphatase was prepared according to Albers and Albers.⁵ Activities were determined according to Bodansky,⁶ except that a different barbital buffer⁷ (pH 9.5) was used and MgCl₂ was added (0.005 mole per liter). Inorganic phosphate was determined photoelectrically by the method of Fiske and Subbarow.⁸ The dialyzed enzyme solutions were reactivated by incubation for 2 hours at 37° with barbital buffer⁷ (pH 9.5), in the absence of magnesium ions.

*Chemical Research Laboratory, Alton Ochsner
Medical Foundation, and the Department
of Biochemistry, Tulane University
School of Medicine
New Orleans*

OTTO SCHALES
GODFREY E. MANN

Received for publication, July 10, 1948

⁵ Albers, H., and Albers, E., *Z. physiol. Chem.*, **232**, 189 (1935).

⁶ Bodansky, A., *J. Biol. Chem.*, **101**, 93 (1933).

⁷ King, E. J., and Delory, G. E., *Biochem. J.*, **33**, 1185 (1939).

⁸ Fiske, C. H., and Subbarow, Y., *J. Biol. Chem.*, **66**, 375 (1925).

THE EFFECT OF VITAMIN DEFICIENCIES UPON THE METABOLISM OF CARDIAC MUSCLE IN VITRO

I. THE EFFECT OF THIAMINE DEFICIENCY IN RATS AND DUCKS*

By ROBERT E. OLSON, OLOF H. PEARSON, O. NEAL MILLER, AND
F. J. STARE

(From the Department of Nutrition, Harvard School of Public Health, and the
Department of Biological Chemistry, Harvard Medical School, Boston)

(Received for publication, April 22, 1948)

Studies with several species have shown that dietary restriction of thiamine results in the fall of tissue cocarboxylase levels and impairment of pyruvate metabolism. Although it appears that cardiac muscle is peculiarly sensitive to deprivation of thiamine (1-3), the degree to which pyruvate utilization in this tissue is impaired in thiamine deficiency has not been ascertained. Sherman and Elvehjem (4) showed that minced heart tissue from polyneuritic chicks gave a decreased oxygen uptake in lactic acid and a decreased rate of lactate removal when compared with controls. Sure and DeWitt (5) observed a similar decrease in the oxygen consumption of minced heart tissue from thiamine-deficient rats in lactate. Muus, Weiss, and Hastings (6) noted that, while the oxygen uptake of ventricular tissue from thiamine-deficient rat heart showed no change from normal in a glucose medium, there was a sharp drop in the oxygen consumption of auricular tissue from deficient rats.

Although Peters and his associates were able to restore the depressed respiration of brain mince from polyneuritic pigeons in a lactate or pyruvate medium by addition of the vitamin *in vitro* (7, 8), Thompson (9) was unable to obtain the same effects with the addition *in vitro* of thiamine to vitamin-deficient heart muscle suspensions.

The object of the present study was to observe the effects of thiamine deficiency upon the metabolism of cardiac tissue, both ventricle and auricle, from rats and ducks. The duck is a fast growing bird particularly suited for these studies because it grows well on a purified diet and requires a relatively short period to develop typical signs of avitaminosis upon withdrawal of a given member of the vitamin B complex (10). The plan of the study was to compare the rates of oxygen consumption, pyruvate disappearance, and lactate formation in cardiac muscle slices from deficient and control animals and birds, to determine the effect of thiamine added *in*

* Supported in part by grants-in-aid from the Life Insurance Medical Research Fund, New York, the Nutrition Foundation, Inc., New York, the Milbank Memorial Fund, New York, and Swift and Company, Inc., Chicago.

vitro to deficient tissues, and to correlate, if possible, the thiamine content of ventricle with its pyruvate utilization.

EXPERIMENTAL

Albino rats and white Pekin ducklings were used in these experiments. Male rats weighing about 100 gm. were divided into control and experimental groups, placed in individual cages, and fed the following diet with and without added thiamine: casein 18 per cent, dextrose 72.6 per cent, salts (11) 4 per cent, corn oil 3 per cent, cod liver oil 2 per cent, cystine 0.2 per cent, choline chloride 0.2 per cent. Crystalline vitamins were added in the following quantities per 100 gm. of diet: riboflavin 600 γ , pyridoxine 400 γ , calcium pantothenate 1.6 mg., niacin 2.0 mg., *p*-aminobenzoic acid 10 mg., inositol 20 mg., and biotin 10 γ . Thiamine was added to the control diet at a level of 400 γ per 100 gm. of diet and both groups were fed *ad libitum*. Newly hatched ducklings were placed in heated, raised bottom cages and fed a commercial mash diet for 7 days, at which time they averaged 150 gm. in weight. They were then divided into control and experimental groups and fed the following diet with and without added thiamine: casein 18 per cent, gelatin 10 per cent, Cellu flour 3 per cent, dextrose 50 per cent, corn oil 10.2 per cent, cod liver oil 2.0 per cent, salts (11) 5 per cent, CaHPO_4 1.0 per cent, choline chloride 0.3 per cent, α -tocopherol in corn oil (10 mg. per ml.) 0.5 per cent. Crystalline vitamins were added in the following quantities per 100 gm. of ration: riboflavin 800 γ , pyridoxine 400 γ , calcium pantothenate 2.0 mg., niacin 4.0 mg., biotin 20 γ , folic acid 100 γ , and 2-methyl-1,4-naphthoquinone 100 γ . Thiamine was added to the control diets at a level of 400 γ per 100 gm. of diet. In order to evaluate the effect of inanition, the control ducks were divided into three groups. One control group was pair-fed with the deficient birds, another group fed *ad libitum*, and a third group fed *ad libitum* through the period required for opisthotonos to develop in the deficient group and then fasted 48 hours. The pair-fed series was injected intraperitoneally with 150 γ of thiamine daily when the food consumption dropped below 20 gm. per day. Daily food consumption and growth records were kept on all birds.

The direct method of Warburg was used for the determination of oxygen consumption. The procedure for the preparation of the rat tissue slices and the composition of the medium used in the rat experiments have been described by Pearson, Hastings, and Bunting.¹ The methods used in the duck experiments were similar except that the medium contained 20 mm per liter of phosphate instead of 7.5 mm per liter and contained no calcium. The period of measurement of oxygen consumption in the duck

¹ Pearson, O. H., Hastings, A. B., and Bunting, H., in preparation.

experiments was 1 hour; in the rat experiments it ranged from 80 to 100 minutes. The gas phase in all experiments was oxygen.

Sodium pyruvate was prepared from freshly distilled pyruvic acid by neutralization with NaHCO_3 to pH 6.5 and crystallization from ethanol. The final concentration of pyruvate in the flask in all experiments was 5 mm per liter. In the rat experiments the pyruvate was present at the time of addition of the tissue; in the duck experiments it was added from the side arm at the time of the first manometric reading (zero time). When glucose was used as a substrate, it was initially present at a level of 0.2 per cent. In studies on the effect of thiamine, the vitamin was initially present in the medium at a concentration of 50 γ per ml. Tissue reactions were stopped at the end of the period of incubation by addition of 0.2 ml. of 100 per cent trichloroacetic acid per flask.

Pyruvate was determined by the direct method of Friedemann and Haugen (12) on the trichloroacetic acid filtrate and the rate of pyruvate disappearance expressed in terms of $-Q_{\text{pyruvate}}$ (microliters of pyruvic acid disappearing per mg. of dry weight of tissue per hour). A determination of the total hydrazones from vessels containing slices of ventricle which had been incubated for 1 hour without added substrate indicated that the amount of keto acids formed in the absence of added pyruvate is very small (of the order of 0.2 mm per flask) and would not introduce an appreciable error in the calculation of pyruvate disappearance from the total hydrazones obtained before and after incubation of heart slices in a medium containing 5 mm per liter of pyruvate originally. Lactate was determined by the method of Barker and Summerson (13) on the same trichloroacetic acid filtrate and the rate of lactate formation expressed in terms of Q_{lactate} (microliters of lactic acid formed per mg. of dry weight of tissue per hour). The latter values were calculated on the *assumption* that the lactate found at the end of the run had been formed during the period from the time of addition of the tissue to the flask at the beginning of the experiment until the time of addition of trichloroacetic acid at the end.

For the determination of the thiamine content of duck ventricle, slices were prepared as for the respiration studies and ground in a Potter homogenizer with 9 volumes of water, boiled, frozen, and finally analyzed for thiamine by the thiochrome method of Wang and Harris (14) after hydrolysis with taka-diastase at pH 4.5.

Results

Calculation of Net Pyruvate Utilization—Since it was evident in the early experiments of this study that the amount of oxygen required to burn to completion the pyruvate disappearing from flasks containing normal heart ventricle slices with added pyruvate was greater than the total oxygen con-

sumption of the slices in the period of measurement, it appeared that pyruvate was being converted to products other than carbon dioxide and water. Analysis for lactate showed that a certain increment of this pyruvate disappearance (about 10 to 20 per cent) could be accounted for by reduction to lactate. An attempt, therefore, was made to correct the total pyruvate disappearance for the fraction reduced to lactate. This corrected value has been called the net $-Q_{\text{pyruvate}}$ and is calculated in the following manner. The value for Q_{lactate} obtained in the absence of added substrate for a given ventricle is subtracted from the value of Q_{lactate} obtained in the presence of added pyruvate to obtain an approximation of the increment of pyruvate converted to lactate per mg. of dry tissue per hour. This quantity is then subtracted from the total $-Q_{\text{pyruvate}}$ to obtain the *net* $-Q_{\text{pyruvate}}$ or the microliters of pyruvate converted to non-lactate products per mg. of dry tissue per hour. It is *assumed* in these calculations that the whole difference in lactate accumulation in the flasks to which no substrate was added and those to which 5 mM per liter of pyruvate were added represents the amount of pyruvate which was reduced to lactate during the incubation. Other experiments, however, have indicated that the amount of pyruvate reduced to lactate is actually less than this because of the gradual oxidation of lactate in vessels to which no substrate was added. In the vessels with added pyruvate no such oxidation occurs. This would tend to diminish somewhat the differences between total pyruvate disappearance and net pyruvate disappearance and, if anything, increase the significance of the differences observed between control and deficient animals. Quantitative data on the pyruvate-lactate relationship in tissue slices from heart as a function of time will be submitted in a separate communication.

Experiments with Rats—Mean growth curves for deficient and control rats are shown in Fig. 1. During the first period of growth decline the deficient rats did not show typical neurological signs. They were then injected subcutaneously with 50 γ of thiamine per rat and this resulted in marked improvement and gain in weight which lasted for a few days. In the subsequent 30 day period the animals again lost weight and appetite and this time showed the typical signs of polyneuritis. The deficient animals were taken for respiration studies during this second period of deficiency and the control animals were used the following week. Table I presents a summary of the data on oxygen consumption, lactate formation, and pyruvate disappearance. These data show that, while the oxygen consumption of thiamine-deficient rat heart ventricle is higher than normal without added substrate, it is slightly, though not significantly, lower in the presence of added pyruvate. Both the total and net pyruvate disappearance, however, are decreased in deficient ventricle. The range of values for

$-Q_{\text{pyruvate}}$ found in the seven deficient rats was from 2.55 to 6.50 with a mean of 5.49 ± 0.45 . In the normal controls fed *ad libitum* the range was from 5.34 to 8.20 with a mean of 7.11 ± 0.50 . The accumulation of lactate was greater in the slices from deficient rats than in the controls, both in the

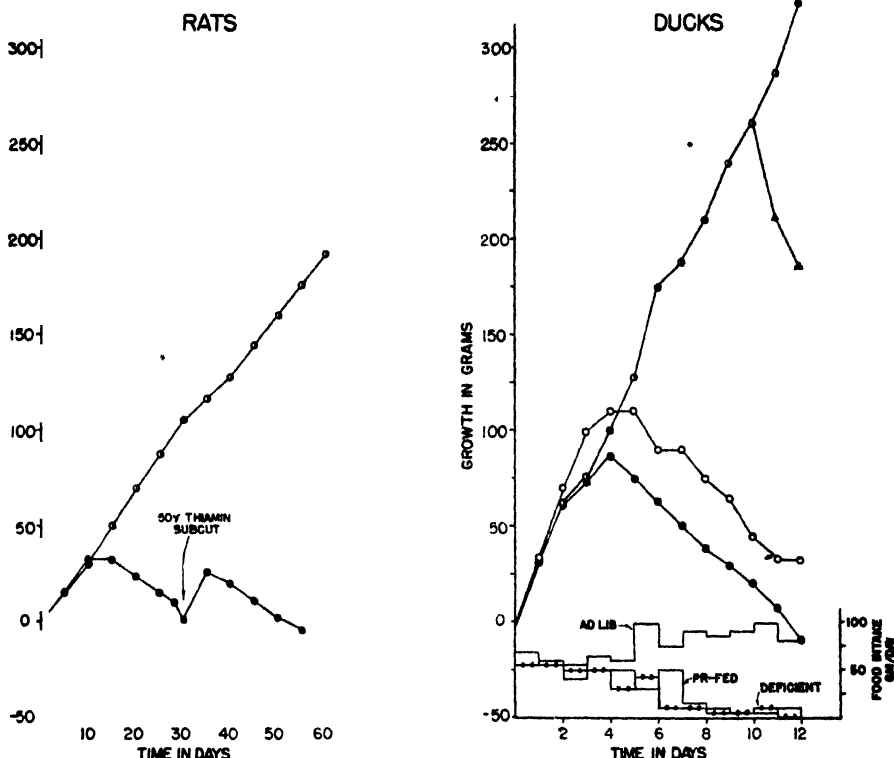


FIG. 1. Growth curves for thiamine-deficient and control rats and ducks. Left, the mean growth of thiamine-deficient rats (●) and normal controls fed *ad libitum* (○) is plotted against the time in days. Right, the mean growth of thiamine-deficient ducks (●), pair-fed normal controls (○), normal controls fed *ad libitum* (●), and normal controls fed *ad libitum*, then fasted from the 10th to 12th days (▲), are plotted against the time in days. Food intake in gm. per day for the ducks is also indicated at the bottom for the thiamine-deficient and for control groups pair-fed and fed *ad libitum*.

presence and absence of added pyruvate, although it is doubtful that the difference is significant in the presence of added pyruvate. The accumulation of lactate in the blood of thiamine-deficient rats has been emphasized by Birch and Harris (2).

The Q_{O_2} values obtained with auricles and ventricles from deficient and

normal rats incubated with added glucose (11.1 mm per liter) are shown in Table II. The finding of a decrease in the Q_{O_2} of auricle from thiamine-deficient rats is in support of the observations of Muus, Weiss, and Hastings (6). The effect of added pyruvate upon the respiration of auricles from normal and thiamine-deficient rats was found, in a few experiments,

TABLE I

Effect of Thiamine Deficiency upon Oxygen Consumption, Pyruvate Disappearance, and Lactate Formation in Heart Ventricle Slices from Rats

Rats	No. of rats	Mean days on diet	Mean weight	Q_{O_2} , no substrate	Q_{lactate} , no substrate	Q_{O_2} , pyruvate, 5 mm per liter	Q_{lactate} , pyruvate, 5 mm per liter	-Pyruvate, 5 mm per liter	Net - Q_{pyruvate}
			gm.						
Deficient	7	50	98	8.88 $\pm 0.52^*$	1.30 ± 0.22	12.86 ± 0.65	4.44 ± 0.95	5.49 ± 0.45	2.69 ± 0.60
Control	5	55	260	7.40 ± 0.59	0.62 ± 0.10	14.58 ± 1.21	3.04 ± 0.43	7.11 ± 0.50	4.63 ± 0.68

* Deviation is expressed as the standard deviation of the mean (standard error).

TABLE II

Effect of Thiamine Deficiency upon Oxygen Consumption of Slices of Ventricle and Auricle from Rats and Ducks

Species	No. of animals	Group	Auricle	Ventricle	Ratio
			Q_{O_2} in glucose, 11.1 mm per liter	Q_{O_2} in glucose, 11.1 mm per liter	Q_{O_2} auricle to Q_{O_2} ventricle
Duck	5	Thiamine-deficient	7.14 $\pm$ 0.24*	7.45 $\pm$ 0.64	0.96
"	8	Pair-fed controls	6.80 $\pm$ 0.38	7.50 $\pm$ 0.27	0.91
"	5	Controls fed <i>ad libitum</i>	6.99 $\pm$ 0.37	7.91 $\pm$ 0.42	0.89
"	4	" " " "	6.24 $\pm$ 0.39	7.69 $\pm$ 0.18	0.81
		then fasted			
Rat	5	Thiamine-deficient	9.52 $\pm$ 0.32	11.32 $\pm$ 0.85	0.84
"	3	Controls fed <i>ad libitum</i>	14.77 $\pm$ 2.25	10.27 $\pm$ 0.72	1.44

* Deviation is expressed as the standard deviation of the mean (standard error).

to parallel the findings in ventricle. The addition of thiamine *in vitro* to deficient ventricle in a single experiment gave an increase in pyruvate disappearance.

Experiments with Ducks—The mean growth curves for the deficient and control groups of ducks are shown in Fig. 1. Loss of weight began in the ducks after 4 days on the deficient diet and acute opisthotonos occurred in

from 7 to 12 days. Birds were taken for respiration studies within 12 hours of their first seizure. When possible, an experiment on a pair-fed control duck was run the same day. The control series fed *ad libitum* was run during the following week. Table III summarizes the data obtained on oxygen consumption, lactate production, and pyruvate utilization with slices of ventricle from deficient and control birds. In contrast to the findings with rats there was no difference in the rate of oxygen consumption by ventricle slices from deficient and control ducks in the absence of added substrate. In the presence of added pyruvate, however, the ventricle of deficient ducks showed a significantly smaller Q_{O_2} than did that of the con-

TABLE III

Effect of Thiamine Deficiency and in Vitro Addition of Thiamine upon the Oxygen Consumption, Pyruvate Disappearance, and Lactate Formation in Heart Ventricle Slices from Ducks

Ducks	No. of ducks	Mean days on diet	Mean weight	Q_{O_2} , no substrate	$Q_{lactate}$, no substrate	Q_{O_2} , pyruvate, 5 mm per liter	$Q_{lactate}$, pyruvate, 5 mm per liter	$-Q_{pyruvate}$, 5 mm per liter	Net $-Q_{pyruvate}$
			gm.						
Deficient	8	9	165	7.88 $\pm 0.24^*$	1.39 ± 0.19	8.41 ± 0.25	2.23 ± 0.17	4.79 ± 0.29	3.95 ± 0.30
" + thia- mine <i>in vitro</i>	8	9	165			9.47 ± 0.17	2.34 ± 0.20	5.46 ± 0.23	4.53 ± 0.32
Pair-fed controls	8	10	208	7.65 ± 0.39	1.04 ± 0.10	9.75 ± 0.32	2.15 ± 0.10	6.04 ± 0.23	4.93 ± 0.24
Controls fed <i>ad libitum</i>	8	11	477	7.55 ± 0.29	0.83 ± 0.10	10.17 ± 0.32	1.71 ± 0.19	6.21 ± 0.18	5.33 ± 0.20
Controls fed <i>ad libitum</i> , then fasted	5	12	389	8.04 ± 0.31	1.82 ± 0.35	9.99 ± 0.22	3.10 ± 0.22	5.89 ± 0.20	4.52 ± 0.27

* Deviation is expressed as the standard deviation of the mean (standard error).

trol group; in fact, the Q_{O_2} of heart ventricle from the deficient birds with added pyruvate is not significantly higher than that obtained without added substrate. The addition of thiamine *in vitro* to slices from deficient ducks gave a significant increase in the oxygen consumption in the presence of added pyruvate (Table III).

The rate of pyruvate disappearance was also depressed in ventricle from the deficient ducks, $-Q_{pyruvate}$ ranging from 1.95 to 5.57 with a mean of 4.79 in the deficient birds compared with a range of 4.78 to 7.90 with a mean of 6.04 in the pair-fed controls. The $-Q_{pyruvate}$ in the other control groups did not differ significantly from 6.04. The *in vitro* addition of thia-

mine to slices from deficient ducks increased the rate of pyruvate disappearance in proportion to the increase in oxygen consumption, about 14 per cent. By averaging the increases in pyruvate disappearance induced by the addition of the vitamin over that of control slices from the same heart, a high degree of significance is revealed for this increase; *viz.*, $\Delta -Q_{\text{pyruvate}} = 0.67 \pm 0.14$ for which $t = 4.71$.²

There was no difference in the accumulation of lactate in slices of ventricle from deficient and pair-fed control ducks with or without added sub-

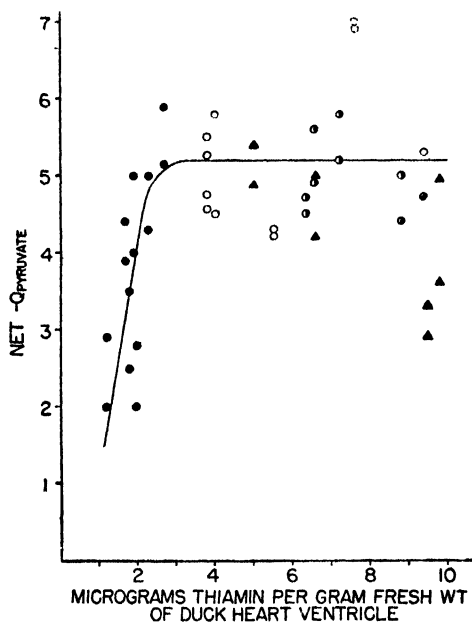


FIG. 2. Relationship between net pyruvate utilization ($\text{net} -Q_{\text{pyruvate}}$) and thiamine content of heart ventricle in ducks. Thiamine-deficient (●), pair-fed normal controls (○), normal controls fed *ad libitum* (◐), and normal controls fed *ad libitum*, then fasted 48 hours (▲).

strate. Lactate accumulation was significantly lower with or without added substrate in the control ducks fed *ad libitum*, however, and significantly higher with or without pyruvate in the ducks fed *ad libitum* which had been fasted 48 hours. In Fig. 2 it may be seen that, despite a high thiamine content of ventricles in this series, the net pyruvate utilization was depressed. These findings would suggest that the state of nutrition with respect to calories preceding the period of inanition and the duration of the

² t is the significance factor of Fisher (Fisher, R. A., *Statistical methods for research workers*, Edinburgh, 4th edition (1932)).

period of inanition are of greater importance in controlling lactate formation *in vitro* than adequacy with respect to thiamine. The addition of thiamine *in vitro* had no effect upon lactate formation or utilization in the presence of pyruvate despite a marked effect upon oxygen consumption and pyruvate disappearance.

The net $-Q_{\text{pyruvate}}$ was significantly lower in the deficient ducks than in the pair-fed or normal controls fed *ad libitum*. The addition of thiamine *in vitro* increased this value as well as the total pyruvate disappearance. The change in net $-Q_{\text{pyruvate}}$ was 0.56 ± 0.10 for which $t = 5.6$.

In Fig. 2 the net $-Q_{\text{pyruvate}}$ values for ventricle slices of all birds observed in these experiments have been plotted against their respective thiamine contents. A good correlation between heart thiamine and pyruvate utilization was found for ventricles containing less than 2.5 γ of thiamine per gm. of fresh weight, although the correlation between the severity of opisthotonos and either heart thiamine or pyruvate utilization was relatively poor. It may be seen from Fig. 2 that a small change in the content of thiamine, *i.e.* from 1.2 to 2.6 γ per gm. of fresh ventricle, resulted in a large increase in net $-Q_{\text{pyruvate}}$, *i.e.* from 2.0 to 5.6. This large range in the pyruvate utilization of heart slices from the deficient ducks tends to raise the mean net $-Q_{\text{pyruvate}}$ value for this group considerably above the value obtained in the "most deficient" bird. It would also appear to indicate that while all of the ducks were sufficiently depleted of thiamine to show neurological signs not all had cardiac tissue sufficiently depleted to show the maximal depression of pyruvate metabolism. Increases in the thiamine content of duck ventricle above 2.5 γ per gm. of fresh tissue had no further influence upon the rate of pyruvate disappearance. The curve in Fig. 2 has the same contour as the one shown by Stumpf, Zarudnaya, and Green (15) for a reconstructed pyruvic acid oxidase system with an apoenzyme from pigeon breast muscle and diphosphothiamine. Swank and Bessey (16) were able to correlate the incidence of electrocardiogram changes in thiamine-deficient pigeons with the thiamine content of their heart muscle. The content of heart thiamine of deficient pigeons without electrocardiogram changes ranged from 1.9 to 2.2 γ per gm. of fresh tissue, of deficient pigeons with definite electrocardiogram changes from 1.5 to 1.7 γ per gm., and of deficient pigeons in cardiac failure from 1.2 to 1.4 γ per gm. In view of our finding of a definite relationship between pyruvate catabolism and thiamine content in ventricle from deficient ducks it is possible that the electrocardiogram changes observed by Swank and Bessey (16) and earlier by others (17) stem from a faulty pyruvate metabolism in the conduction system.

Table IV shows a tabulation of the range and the mean of the thiamine content per gm. of fresh weight of duck ventricle for the deficient and con-

trol groups. The normal values are somewhat higher than those reported by Ochoa and Peters (18) and by Swank and Bessey (16) for normal pigeon heart, but nearly identical with the values reported by Mitchell and Isbell (19) for normal rat heart. The mean of the deficient group is significantly below that of any control group.

A comparison of the rates of oxygen consumption by auricle and ventricle slices from deficient and control groups of rats and ducks in phosphate-saline containing 0.2 per cent glucose is presented in Table II. It may be seen that the auricles from thiamine-deficient ducks do not show the same depression in Q_{O_2} shown by auricles from thiamine-deficient rats.

TABLE IV

Variation in Thiamine Content of Ventricle in Thiamine-Deficient and Control Ducks

Group	No. of determinations	Thiamine content, γ per gm. fresh ventricular slice	
		Mean	Range
Deficient.	7	$1.86 \pm 0.18^*$	1.15-2.58
Pair-fed controls	6	5.44 ± 0.94	3.66-9.10
Controls fed <i>ad libitum</i>	4	6.85 ± 1.38	4.74-9.41
" " " " then fasted	4	6.94 ± 0.53	6.05-8.44

* Deviation is expressed as the standard deviation of the mean (standard error).

DISCUSSION

The results of this study indicate that the pyruvate utilization by cardiac muscle from rats and ducks is depressed in thiamine deficiency and that the addition of thiamine *in vitro* tends to restore pyruvate utilization to normal. Analyses of deficient and normal duck heart ventricle for thiamine, furthermore, have shown that a good correlation exists between the thiamine content and the ability of the tissue to utilize pyruvate. Inanition comparable to that sustained by the deficient ducks in pair-fed normal controls was shown not to influence significantly the rate of pyruvate utilization.

The failure to find any difference in the rate of oxygen consumption by heart ventricle from normal and thiamine-deficient ducklings *without added substrate* is in agreement with the experiments of others on tissues from polyneuritic birds (20, 21). A significant increase in the oxygen consumption of heart ventricle from thiamine-deficient rats over that of controls, however, was observed. The finding that the addition of thiamine *in vitro* will stimulate the respiration of deficient ventricle slices, the so called "catalorulin effect" first shown by Peters and coworkers (20) in pigeon brain, is of interest. Sherman and Elvehjem (4) have shown a similar effect in studies of minced heart suspensions from thiamine-deficient chicks incubated with

lactate. The relationship between pyruvate disappearance and oxygen consumption in the duck heart ventricle is similar to that reported for pigeon brain by Peters and Thompson (8) and would indicate that under our conditions not all of the pyruvate disappearing to non-lactate products is burned to CO_2 and H_2O . In rat ventricle, however, the relationship appears superficially to be in better agreement with theory for quantitative combustion, as shown by the studies of Krebs and Eggleston (22) on pigeon breast muscle and those of Gibson and Long (23) on dialyzed ox heart suspensions. Actually, the oxygen consumption of rat ventricle more than accounts for the complete oxidation of the calculated net $-Q_{\text{pyruvate}}$ but an error in the correction for lactate formation from pyruvate discussed earlier would tend to raise the observed net $-Q_{\text{pyruvate}}$ to the theoretical value of $Q_{\text{O}_2}/2.5$. This observation of a correspondence between oxygen consumption and pyruvate disappearance in heart muscle slices suggests but does not constitute *proof* that only pyruvate is being oxidized under the conditions of our experiments. The disparity between net $-Q_{\text{pyruvate}}$ and $Q_{\text{O}_2}/2.5$ was exaggerated in the thiamine-deficient rats.

Fasting of rapidly growing ducks fed *ad libitum* for 48 hours resulted in an increase in the accumulation of lactate by ventricle slices incubated with or without added pyruvate and a decrease in the conversion of added pyruvate to non-lactate products. Rosenthal (24) has reported a decreased utilization of pyruvate and lactate in liver slices from starved animals and Lipschitz, Potter, and Elvehjem (25) have reported that liver and kidney but not brain homogenates from normal chicks fasted 44 to 196 hours showed a reduction in pyruvate removal comparable to that obtained in polyneuritic chicks. The administration of glucose restored utilization of pyruvate to normal in the fasted chicks but not in the deficient ones. In our experiments fasting had no effect upon total pyruvate removal but appeared to influence the partition of pyruvate between lactate and non-lactate products in normal ducks fasted 48 hours; there was no significant effect of inanition upon either total or net pyruvate disappearance in the ducks whose food intake had been curtailed stepwise in conformity to the intake of the deficient birds. It would appear, therefore, that brain and heart are more resistant to the effects of fasting upon pyruvate metabolism than are kidney and liver.

The differences in the effect of thiamine deficiency upon the respiration of auricles in the duck and in the rat may possibly be related to the period of induction of polyneuritis in each species. In the rat the mean period was 7.5 weeks; in the duck it was 9 days. The finding of fibrotic lesions in the auricles of rats (26) and of pigs (27) after relatively long deficiency periods may indicate that the reduction of Q_{O_2} in the auricles of thiamine-deficient rats is due to a decrease in active protoplasm through fibrosis and

not to a biochemical lesion. The shortness of the deficiency period for the duck would preclude the development of fibrosis.

The demonstration of a decrease in the ability of cardiac muscle to metabolize pyruvate in thiamine deficiency is in further support of a recent study by Randles *et al.* (28) on dogs in which it was found that hearts from thiamine-deficient dogs removed less pyruvate from the blood *in vivo* than did hearts from normal controls.

SUMMARY

1. The oxygen consumption of heart ventricle slices without added substrate is not affected by thiamine deficiency in ducks but is increased by thiamine deficiency in rats.

2. The oxygen consumption of heart ventricle slices with added pyruvate (5 mM per liter) is decreased by thiamine deficiency in ducks but is unaffected by thiamine deficiency in rats.

3. The rate of pyruvate disappearance in heart ventricle and the rate of its conversion to non-lactate products are decreased by thiamine deficiency in both species.

4. The addition of thiamine *in vitro* to heart ventricle slices from deficient ducks increases both the oxygen consumption and the pyruvate disappearance.

5. The rate of pyruvate utilization in duck ventricle increases with the thiamine content up to a level of 2.5 γ per gm. of fresh ventricle. Further increases in thiamine content have no further effect upon pyruvate utilization.

6. A comparison of the rates of lactate accumulation with and without added pyruvate in normal and thiamine-deficient rats and ducks would indicate that inanition is more effective in increasing lactate formation than is thiamine deficiency.

7. The depression in the oxygen consumption of auricular muscle seen in thiamine-deficient rats was not observed in thiamine-deficient ducks. An explanation for this species difference is suggested.

We are greatly indebted to Miss Charlotte Thrall for technical assistance in carrying out the experiments of this study. We are also indebted to Merck and Company, Inc., Rahway, New Jersey, the Corn Industries Research Foundation, New York, and the Sheffield Farms Company, Inc., New York, for generous supplies of materials used in these studies.

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THE EFFECT OF VITAMIN DEFICIENCIES UPON THE METABOLISM OF CARDIAC MUSCLE IN VITRO

II. THE EFFECT OF BIOTIN DEFICIENCY IN DUCKS WITH OBSERVATIONS ON THE METABOLISM OF RADIOACTIVE CARBON-LABELED SUCCINATE*

By ROBERT E. OLSON, O. NEAL MILLER, YALE J. TOPPER, AND F. J. STARE

(From the Department of Nutrition, Harvard School of Public Health, and the Department of Biological Chemistry, Harvard Medical School, Boston)

(Received for publication, April 22, 1948)

The gross effects of biotin deficiency in bacteria, animals, poultry, and man have been studied in detail by a number of investigators. They range from growth failure in all species to dermatitis, alopecia, neuromuscular imbalance, anemia, spectacle eye, and thymus and testis atrophy in rats (1-4), poor feathering, dermatitis, and perosis in poultry (5-9), and changes in skin color, somnolence, glossitis, and electrocardiographic changes in man (10, 11). Data bearing on the underlying biochemical defects in biotin deficiency, however, have only recently been forthcoming.

In 1942 Pilgrim, Axelrod, and Elvehjem (12) showed that homogenates of liver from biotin-deficient rats oxidized pyruvate more poorly than did those from normal controls and, in 1944, Summerson, Lee, and Partridge (13) reported that the addition of biotin to liver slices from biotin-deficient rats *in vitro* resulted in increased lactate utilization. In 1943 Burk and Winzler (14) suggested that biotin might be concerned with carbon dioxide transfer. Koser, Wright, and Dorfman (15) and Stokes, Larsen, and Gunness (16) have reported that aspartate synthesis by bacteria is depressed in the absence of biotin, and Lardy, Potter, and Elvehjem (17) and Shive and Rogers (18) have presented data which account for this depressed aspartate synthesis on the basis of a failure to fix carbon dioxide in oxalacetate. Lichstein and Umbreit (19) have demonstrated recently the need for biotin in the decarboxylation of oxalacetate by *Escherichia coli*, and Ochoa *et al.* (20) have shown that the content of triphosphopyridine nucleotide-specific malate dehydrogenase-decarboxylase is lowered in biotin-deficient turkey liver.

Recent studies of the metabolism of cardiac muscle have shown that pyruvate utilization *in vitro* is depressed by thiamine deficiency in rats and

* Supported in part by grants-in-aid from the Life Insurance Medical Research Fund, New York, Swift and Company, Inc., Chicago, the Nutrition Foundation, Inc., New York, the Milbank Memorial Fund, New York, and in part (Y. J. T.) by a contract between Harvard University and the Office of Naval Research.

ducks (21) and by pantothenic acid deficiency in rats (22). It occurred to us that if biotin functions in metabolism to facilitate the reversible β -carboxylation of pyruvate to oxalacetate the utilization of pyruvate as well as other members of the tricarboxylic cycle might be depressed in tissues from biotin-deficient birds. To test this hypothesis we have determined the rate of oxygen consumption of slices of heart muscle from biotin-deficient and control ducks without added substrate and with added pyruvate and succinate, the rate of pyruvate disappearance in the presence of added pyruvate, and the rate of carbon dioxide production from succinate. The effect of pair-feeding normal controls, the effect of adding biotin to deficient tissues *in vivo* and *in vitro*, and the comparative effects of deficiency upon the respiration of auricle and ventricle were also studied. A preliminary report of these experiments has been made (23).

EXPERIMENTAL

White Pekin ducklings 2 days old, averaging 50 gm. in weight, were divided into experimental and control groups and placed in heated, raised bottom cages. The control groups were fed the following diet: casein 18 per cent, gelatin 10 per cent, Cellu flour 3 per cent, dextrose 50 per cent, corn oil 10.2 per cent, cod liver oil 2.0 per cent, salts (24) 5 per cent, CaHPO_4 1 per cent, choline chloride 0.3 per cent, α -tocopherol in corn oil (10 mg. per ml.) 0.5 per cent. Supplementary crystalline vitamins were added in the following quantities, expressed in micrograms per 100 gm. of ration: thiamine 400, riboflavin 800, pyridoxine 400, nicotinic acid 4000, calcium pantothenate 2000, folic acid 100, menadione 100, and biotin 20. The experimental series was fed the same diet supplemented with 20 per cent dried raw egg white at the expense of carbohydrate and no biotin. To evaluate the effect of the partial inanition of biotin deficiency, several groups of control ducks were pair-fed either a deficient diet with parenteral biotin or the control diet. Other groups of control ducks were fed the control diet *ad libitum*.

Deficient ducks were taken for respiration studies after their growth had been arrested and some weight loss had occurred. The birds were killed by decapitation, the heart quickly excised, chilled on cracked ice, and then immersed in iced, oxygenated saline-phosphate solution. The auricles and ventricles of the chilled heart were removed, sliced to a thickness of 0.5 to 0.7 mm., placed in standard Warburg flasks containing potassium hydroxide in the center well, gassed with oxygen, equilibrated at 37°, and the rate of oxygen consumption determined. Each flask contained a total volume of 3.0 ml. of phosphate-saline at pH 7.4 of the following composition: NaCl 0.119 M, KCl 0.004 M, MgCl_2 0.0005 M, and $\text{Na}_2\text{HPO}_4\text{-NaH}_2\text{PO}_4$ 0.020 M. When biotin was added *in vitro* it was present in the medium initially and

allowed to react with tissue components for 30 minutes before the addition of substrate. For study of the effects of biotin *in vivo*, the vitamin was injected intraperitoneally into deficient ducks in amounts of 45 γ per bird per day.

Substrates were added from the side arm at zero time. Sodium pyruvate was prepared from redistilled pyruvic acid which was neutralized to pH 6.5 with bicarbonate and then crystallized from ethanol. The final concentration of pyruvate in the Warburg flask after dumping was 5 mm per liter. The non-isotopic succinate was chemically pure sodium succinate. Isotopic succinic acid containing C^{14} distributed between its two carboxyl groups was prepared from lithium acetylide by carbonation with $C^{14}O_2$ and subsequent reduction. The final product melted at 189–190° and contained about 440,000 counts per minute per mm. For respiration studies this was diluted with non-isotopic succinate to yield an activity of about 10,000 counts per minute per mm. The final concentration of succinate in the Warburg flask was 10 mm per liter.

Oxygen uptake was measured for 1 hour. At the end of this period tissue reactions were stopped by addition of 0.2 ml. of 100 per cent trichloroacetic acid. In the case of the flasks containing radioactive succinate this addition was made in a closed system so that the $C^{14}O_2$ liberated from the buffer and tissue would be absorbed by the alkali of the center well. For determinations of the amount of succinate oxidized the contents of the center well were pipetted with washings into a 15.0 ml. centrifuge tube (a filter paper in the center well was generally *not* used in the succinate experiments because it was shown that even assiduous washing of the alkali-soaked filter paper would leave 5 to 10 per cent of the carbon dioxide in the paper) and precipitated with $BaCl_2$. The barium carbonate was centrifuged and washed with water until free of alkali and finally with ethanol and then plated on a tared, round, stainless steel cup having a central depression 1.5 mm. deep and 1.6 sq. cm. in area. The samples were counted with an end window Geiger counter and corrected for background, self-absorption, and counter sensitivity. The accuracy of the counting as determined by checks with samples of known activity was ± 5 per cent.

Pyruvate was determined by the direct method of Friedemann and Haugen (25) and lactate by the method of Barker and Summerson (26) on trichloroacetic acid filtrates of the Warburg fluid after incubation. Final dry weight of the tissue slices was determined by heating to constant weight in an oven at 110°. In the protocols, the rate of oxygen consumption has been expressed as Q_{O_2} (microliters of O_2 used per mg. of dry weight of tissue per hour), pyruvate disappearance as $-Q_{\text{pyruvate}}$ (microliters of pyruvate disappearance per mg. of dry weight of tissue per hour, 1 micromole of pyruvate being equivalent to 22.4 microliters), and lactate formation as Q_{lactate} .

The net pyruvate disappearance, *i.e.* the conversion of pyruvate to non-lactate products, is expressed as *net* $-Q_{\text{pyruvate}}$ (21).

Results

About 70 ducklings were used in this study. Typical growth curves for deficient, pair-fed controls, and controls fed *ad libitum* are shown in Fig. 1. Each point on the growth curve represents the mean of ten ducks. In this series of experiments it was invariably found that the experimental group receiving egg white grew more rapidly for the first 6 to 7 days and then lagged behind the control groups, reaching a plateau at about 200 gm. The food consumption, which is also plotted for all groups in Fig. 1, dropped to 30 gm. per day for the experimental group after the 1st week and remained at or about that level for the duration of the experiment. Poor feathering and baldness were noticed as early as 4 to 6 days in the experimental groups and occurred in some 90 per cent of the deficient ducks. Perosis was observable between the 8th and 24th days and occurred in 70 per cent of the deficient ducks. Mortality in the deficient group ranged from 20 to 30 per cent. The pair-fed control groups began to lag in growth after 10 days on their regimen and generally reached a plateau at a body weight of 300 to 350 gm. These birds were continually famished and consumed their food allowance in 30 to 45 minutes daily. Either pair-feeding the control diet or pair-feeding the deficient diet plus intraperitoneal injections of 20 γ of biotin per duck per day gave the same growth curve. In general, the hearts of the deficient ducks were paler, larger, and the myocardium thicker than in those from controls fed *ad libitum* or pair-fed.

The effect of biotin deficiency upon the respiration of slices of ventricle is shown in Table I. Mean data on oxygen consumption, lactate formation, and pyruvate utilization for deficient, cured, and control ducks are presented. All values are depressed in biotin deficiency. When pair-fed controls are used as the standard of comparison, the Q_{O_2} of heart ventricle from biotin-deficient ducks was reduced 43 per cent without added substrate and 35 per cent with added pyruvate. Pyruvate utilization was decreased 48 per cent. When compared to slices from pair-fed ducks the lactate accumulation expressed as Q_{lactate} was 15 per cent of normal in the absence of added substrate and 25 per cent of normal in the presence of pyruvate at 5 mm per liter. Compared with values from ducks fed *ad libitum*, the above values become 39 per cent and 52 per cent respectively. The elevation of lactate formation in the pair-fed birds is reminiscent of our findings in normal ducks fasted for 48 hours (21) and suggests that the pair-fed ducks in this series are effectively fasted by the feeding of suboptimal food allowances which are almost immediately consumed. Time studies of

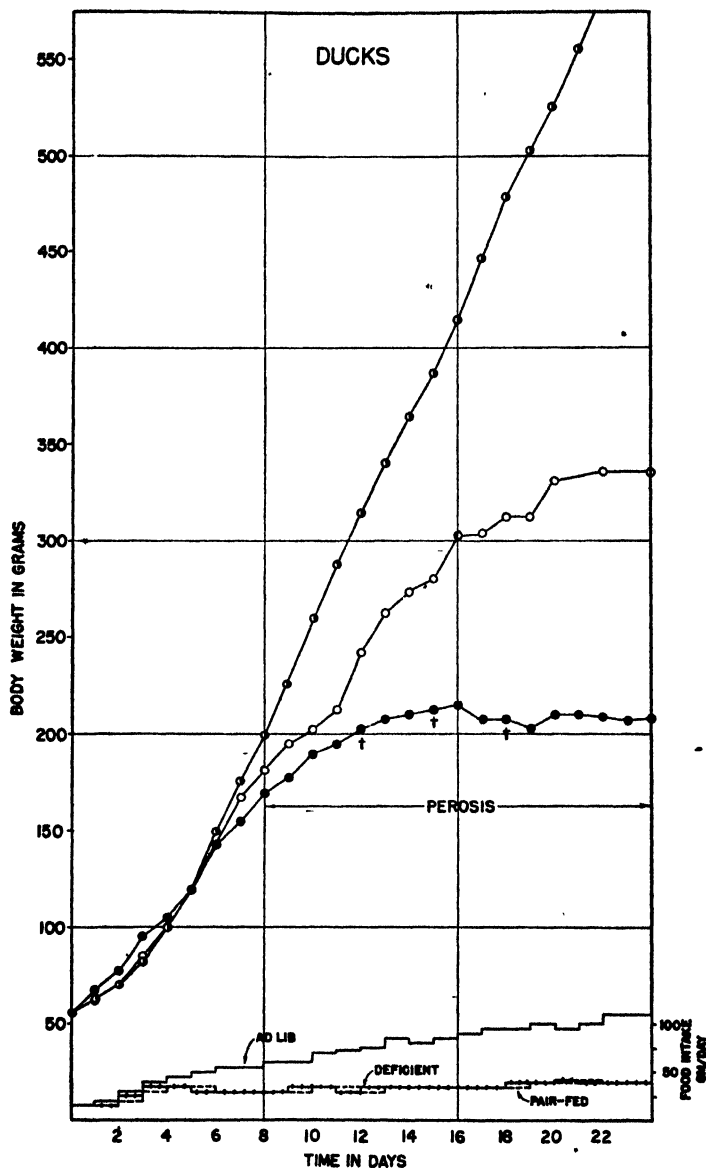


FIG. 1. Growth curves for biotin-deficient, pair-fed normal control, and control ducks fed *ad libitum*. The mean body weight in gm for groups of ten biotin-deficient (●), pair-fed control (○), and controls (◐) fed *ad libitum* is plotted against the time in days. The crosses indicate death. Perosis was observable after the 8th day. The food intake in gm. per day per duck is plotted for these groups across the bottom.

the rate of lactate formation in ventricular muscle from normal ducks and those fed *ad libitum* indicate that the lactate accumulation is a product of initial glycolysis and subsequent oxidation in slices without added pyruvate and the product of initial glycolysis plus further reduction of pyruvate in the tissues with added pyruvate (27). The data obtained with biotin deficiency would seem to indicate that the initial glycolysis is decreased.

When deficient birds are injected with biotin intraperitoneally at a level of 45 γ per bird per day, there is restoration of respiration to normal within 24 hours of the injection. The data on cured ducks shown in Table I were obtained from three deficient ducklings showing all the features of the biotin deficiency syndrome and injected with biotin from 1 to 7 days. One

TABLE I

Effect of Biotin Deficiency upon Oxygen Consumption, Pyruvate Utilization, and Lactate Formation in Heart Ventricle Slices from Ducklings

Group	No.* of ducks	Days on diet	Mean weight	QO_2 , no substrate	QO_2 , pyruvate, 5 mm per liter	$Q_{lactate}$, no substrate	$Q_{lactate}$, pyruvate, 5 mm per liter	$-Q_{pyruvate}$	Net $-Q_{pyruvate}$
Deficient	9	18	218	4.54	7.02	0.24	0.82	3.12	2.54
		± 1 †	± 16	± 0.29	± 0.26	± 0.06	± 0.10	± 0.22	± 0.21
Pair-fed controls	6	24	374	7.96	10.73	1.64	3.11	6.42	4.92
		± 1	± 35	± 0.21	± 0.30	± 0.14	± 0.15	± 0.32	± 0.38
Controls fed <i>ad libitum</i>	8	18	504	7.25	10.10	0.62	1.58	6.01	5.08
		± 1	± 35	± 0.34	± 0.33	± 0.05	± 0.15	± 0.34	± 0.31
Deficient + biotin intraperitoneally	3	18	309	6.95	9.74	0.45	1.41	5.37	4.40
		± 2	± 100	± 0.22	± 0.42	± 0.05	± 0.23	± 0.26	± 0.35

* Duplicate determinations made on each duck.

† All deviations are the standard error of the mean

bird was killed after 1 day, one after 2 days, and one after 7 days. The one allowed to go for 7 days showed resumption of growth at a slope paralleling the normal controls fed *ad libitum* and refeathering. It may be seen that the oxygen consumption with and without added pyruvate and pyruvate utilization in these cured ducks are restored to levels not significantly different from those of normal controls.

Table II shows the results of studies on the oxidation of succinate by these same groups of biotin-deficient, cured, and control ducks. The oxygen consumption by ventricle from biotin-deficient ducks in the presence of succinate is decreased below the values found for control birds and is restored by injections of biotin. Column 4 of Table II lists the radioactivity recovered in the center well of the Warburg vessels after incubation.

Column 5 of Table II gives the rate of oxidation of succinate in terms of Q_{CO_2} (succinate carboxyl). These values were calculated from the initial total quantity and activity of the succinate carboxyl CO_2 and the final activity of the respiratory CO_2 collected from the center well; *viz.*, each flask contained 3.0 ml. of buffer having a succinate concentration of 10 mm per liter. The total succinate per flask was thus 30 micromoles and the total succinate carboxyl CO_2 , 60 micromoles. The total radioactivity of the succinate

TABLE II

Comparative Rates of Succinate Oxidation in Biotin-Deficient, Cured, and Control Duck Ventricle As Measured with Isotopic Succinate Labeled in Carboxyl with C^{14}

Group	No. of determinations	Q_{O_2} , succinate, 10 mm per liter	$C^{14}O_2$ produced during incubation (1 hr.)		Per cent of values obtained in pair-fed controls	
			Counts per min per 100 mg. dry weight tissue per hr. (4)	Q_{CO_2} , succinate carboxyl (5)	Q_{O_2} , succinate, 10 mm per liter (6)	Q_{CO_2} , succinate carboxyl (7)
(1)	(2)	(3)	(4)	(5)	(6)	(7)
Deficient	18	13.04 $\pm$ 0.49*	56 $\pm$ 4	2.55 $\pm$ 0.18	72 $\pm$ 2	45 $\pm$ 3
Pair-fed controls	11	18.01 $\pm$ 0.68	126 $\pm$ 7	5.74 $\pm$ 0.31	100 $\pm$ 4	100 $\pm$ 5
Controls fed <i>ad libitum</i>	14	17.13 $\pm$ 0.41	90 $\pm$ 4	4.17 $\pm$ 0.20	95 $\pm$ 2	73 $\pm$ 4
Deficient + biotin intraperitoneally	6	15.76 $\pm$ 0.45	132 $\pm$ 10	6.04 $\pm$ 0.47	88 $\pm$ 3	105 $\pm$ 8

* All deviations are the standard error of the mean.

carboxyl was 300 counts per minute per flask. The period of incubation was 1 hour. From this it follows

$$\text{Activity of center well, } CO_2, \text{ counts per min. per flask} \times 60 \times 22.4$$

300

$$\times \text{dry weight of tissue, mg.} = Q_{CO_2} \text{ (succinate carboxyl)}$$

The Q_{CO_2} (succinate carboxyl) is thus the number of microliters of succinate carboxyl carbon dioxide produced per mg. of dry weight of tissue per hour. Since only the carboxyl groups of the isotopic succinate are labeled, and since the known pathways for succinate oxidation make possible removal of one carboxyl without the other, the Q_{CO_2} (succinate carboxyl) values obtained in the above manner are not indicative of the number of moles of succinate undergoing oxidation or the extent of the oxidation. If one wished to *assume* for the sake of an approximation that the liberation of one carboxyl CO_2 insured the *complete* combustion of succinate under our conditions,

then the rate of succinate combustion in microliters (micromoles $\times 22.4$) per mg. of dry weight per hour would equal one-half the $Q_{\text{CO}_2(\text{succinate carboxyl})}$.

It is of interest to note (Table II, Columns 6 and 7) that biotin deficiency depresses CO_2 production from succinate in heart muscle more than it lowers oxygen consumption in the presence of succinate. Conversely, the administration of biotin to deficient ducks restores CO_2 production from succinate more completely than it restores oxygen consumption in the presence of succinate. These data are in support of the idea that the activity of biotin is concerned with decarboxylation reactions. If one calculates the $-Q_{\text{succinate}}$ values for normal and deficient hearts from the $Q_{\text{CO}_2(\text{succinate carboxyl})}$ values on the basis of assumptions outlined in the preceding paragraph, and then calculates the equivalent oxygen consumption from $-Q_{\text{succinate}}$ by multiplying by 3.5, it would appear that, while

TABLE III

Effect of Biotin Addition in Vitro upon Oxygen Consumption, Pyruvate Utilization, and Lactate Formation in Slices of Heart Ventricle from Biotin-Deficient Ducks

Ventricle slices	No. of determinations	Q_{O_2} , no substrate	Q_{O_2} , pyruvate, 5 mm per liter	Q_{O_2} , succinate, 10 mm per liter	Q_{lactate} , no substrate	Q_{lactate} , pyruvate, 5 mm per liter	$-Q_{\text{pyruvate}}$	$\text{Net } -Q_{\text{pyruvate}}$
Deficient	6	5.13 $\pm 0.65^*$	5.36 ± 0.68	12.34 ± 1.38	0.43 ± 0.12	0.87 ± 0.09	3.58 ± 0.13	3.14 ± 0.18
“ + biotin, 5 γ per ml.	6	4.02 ± 0.55	6.78 ± 0.89	11.38 ± 1.85	0.32 ± 0.07	0.71 ± 0.07	3.97 ± 0.28	3.56 ± 0.30
Normal controls	10	7.03 ± 0.34	9.82 ± 0.32	17.37 ± 0.52	0.75 ± 0.12	1.75 ± 0.21	6.01 ± 0.38	5.01 ± 0.36

* All deviations are the standard error of the mean.

succinate combustion in normal heart ventricle accounted for 50 per cent of the observed oxygen consumption, in heart slices from biotin-deficient ducks it accounted for only 34 per cent. It is probable that the succinoxidase system which oxidizes succinate to fumarate (and other systems which oxidize fumarate to oxalacetate) accounts for a fair proportion of the extra oxygen consumption in both the normal and deficient tissues. It is of some interest that the succinate utilization of heart ventricle from control ducks fed *ad libitum* is below that of pair-fed controls.

Attempts to remedy the respiration defects in ventricle from biotin-deficient ducks by adding the vitamin *in vitro* and incubating 30 minutes before adding substrates failed, as indicated by Table III. There were no significant changes in oxygen consumption in the absence and presence of added pyruvate, in the presence of succinate, in pyruvate utilization, or in lactate formation. The only positive change was in the oxygen con-

sumption in the presence of pyruvate, a change from 5.36 ± 0.68 in the absence of biotin to 6.78 ± 0.89 in the presence of biotin, a difference of 1.42 for which $t = 1.3$, which is not significant. Normal control slices from ducks fed *ad libitum* run with this series showed normal values not different from the previous series of controls.

Table IV presents a comparison of the effects of biotin deficiency upon the respiration of auricle and ventricle slices taken from the same heart in a series of ducks. The same decreases in Q_{O_2} and pyruvate utilization were observed for both auricle and ventricle. In general, the changes were parallel in both auricle and ventricle for all of the measurements made with the exception of oxygen consumption in the presence of pyruvate, which seemed to be depressed in auricle more than in ventricle. The degree of change from normal in this series of deficient ducks was not quite as

TABLE IV

Comparative Rates of Oxygen Consumption and Pyruvate Utilization by Slices of Auricle and Ventricle from Normal and Biotin-Deficient Ducks

	Group No. of determinations	Deficient 12	Control 6
Auricle	Q_{O_2} (no added substrate)	5.65 ± 0.24	7.96 ± 0.71
	" (pyruvate, 5 mm per liter)	5.83 ± 0.31	10.92 ± 0.35
	Net $-Q_{\text{pyruvate}}$	3.40 ± 0.31	5.25 ± 0.52
Ventricle	Q_{O_2} (no added substrate)	6.08 ± 0.36	8.20 ± 0.75
	" (pyruvate, 5 mm per liter)	7.80 ± 0.43	10.85 ± 0.55
	Net $-Q_{\text{pyruvate}}$	3.62 ± 0.23	5.38 ± 0.01
Ratio, auricle to ventricle	Q_{O_2} (no added substrate)	0.93 ± 0.07	0.97 ± 0.10
	" (pyruvate, 5 mm per liter)	0.75 ± 0.05	1.01 ± 0.04
	Net $-Q_{\text{pyruvate}}$	0.94 ± 0.15	0.98 ± 0.07

marked as in earlier series, although the results are still significantly different from normal. This group of deficient birds was *older* and it has been found in two or three cases that birds which survive more than 24 days on the deficient diet give values for oxygen consumption and pyruvate utilization for heart ventricle which are closer to normal than are those obtained on ducks showing gross deficiency signs at 12 to 16 days. The nature of this accommodation to deficiency disease is being explored.

DISCUSSION

The finding of a deranged pyruvate and succinate metabolism in cardiac muscle slices from biotin-deficient ducks adds to the growing weight of evidence assigning to biotin the rôle of a respiratory catalyst. As early as 1933, Allison, Hoover, and Burk (28) showed that a heat-stable water-

soluble factor from yeast which they named coenzyme R and which was later identified with biotin (29, 30), would stimulate the respiration of the *Rhizobium trifolii* from legume nodules 100-fold. More recently, Burk, Winzler, and du Vigneaud (31) have pointed out that the respiration of biotin-deficient yeast is markedly depressed and the addition of biotin stimulates both fermentation and respiration. The finding of a depressed *endogenous* oxygen consumption in ventricle slices from biotin-deficient ducks is, therefore, not unexpected. The utilization of pyruvate by both ventricle and auricle has been found to be depressed in biotin-deficient ducks. This effect has been previously observed in liver tissue from biotin-deficient rats by Pilgrim, Axelrod, and Elvehjem (12) and by Summerson, Lee, and Partridge (13).

Recent studies mentioned previously (15-18) have implicated biotin in the reversible β -carboxylation of pyruvate to oxalacetate. Although the Wood-Werkman reaction has not been shown to occur in heart muscle, fixation of CO_2 in oxalosuccinate by heart muscle extracts has been shown by Ochoa (32). Presumably, fixation of CO_2 in oxalacetate also occurs in heart muscle and experiments to verify this are under way at present. The production of CO_2 from succinate is significantly slowed in ventricle from biotin-deficient ducklings. The conversion of pyruvate to non-lactate products is decreased to almost the same quantitative extent, *i.e.* 50 per cent. If the fixation of CO_2 by pyruvate is a prerequisite for the maintenance of adequate levels of oxalacetate for condensation of C_2 or C_3 radicals from pyruvate and their ultimate oxidation by way of the tricarboxylic acid cycle, a loss of β -carboxylase activity would explain both the decreased CO_2 production from succinate and the decreased pyruvate utilization in cardiac ventricle slices from biotin-deficient ducks. The decreased lactate accumulation observed in these slices is more difficult to explain. That biotin has a single function in metabolism is doubtful in view of the studies of Lichstein and Umbreit (33) on the deaminase content of biotin-deficient *Escherichia coli*, of McHenry and Gavin (34) on synthesis of fatty acids in rats supplemented with biotin, and of Potter and Elvehjem (35) on oleic acid-biotin interrelationships in *Lactobacillus arabinosus*.

The mechanism of the action of biotin is not clear. Although biotin appears to influence the reversible β -carboxylation of pyruvate, other co-factors are also involved. Vennesland, Evans, and Altman (36) found stimulation of CO_2 fixation by pigeon liver β -carboxylase in the presence of adenosine triphosphate but stimulation of the reverse reaction in the presence of triphosphopyridine nucleotide. Cheldelin *et al.* (37) report that biotin is more firmly bound in tissue combination than any other vitamin. Our studies with the addition of biotin to deficient tissues *in vitro* indicate that the vitamin is not easily converted to an active form. The relative efficacy of biotin upon deficient ducks *in vitro* and *in vivo* parallels, however,

the effects of pantothenic acid (22) which is conjugated to a relatively complex coenzyme. Ochoa *et al.* have found no biotin by a microbiologic technique in their preparations of malate dehydrogenase-decarboxylase and entertain the possibility that biotin may in some way stimulate enzyme formation. The resolution of the mechanism of biotin activity awaits further investigation.

SUMMARY

1. The oxygen consumption of heart ventricle slices from biotin-deficient ducks in the presence and absence of pyruvate (5 mM per liter) and in the presence of succinate (10 mM per liter) was decreased 35, 43, and 28 per cent respectively from that of ventricle slices from pair-fed normal controls. The values for ventricle from controls fed *ad libitum* were not significantly different from those of the pair-fed controls.

2. The accumulation of lactate in ventricle slices from biotin-deficient ducks in the presence and absence of pyruvate was decreased to about 20 per cent of the values from pair-fed controls and to about 45 per cent of those from controls fed *ad libitum*. The higher lactate formation in pair-fed ducks appears to be related to their state of partial inanition.

3. The conversion of pyruvate to non-lactate products was reduced 48 per cent in ventricle slices from biotin-deficient ducks.

4. $C^{14}O_2$ production from carboxyl-labeled succinate in heart ventricle from biotin-deficient ducks was decreased 55 per cent from that of pair-fed controls.

5. The prior intraperitoneal administration of biotin to deficient ducks restored the respiration and pyruvate utilization of heart ventricle slices essentially to normal.

6. The incubation of deficient heart ventricle slices with biotin *in vitro* had no significant effect upon their respiration or pyruvate utilization.

7. The respiration and pyruvate utilization of auricular and ventricular heart muscle, in general, were depressed to the same extent in biotin-deficient ducks.

We wish to thank Miss Charlotte Thrall for technical assistance. Our thanks are also due to Merck and Company, Inc., Rahway, New Jersey, the Corn Industries Research Foundation, New York, and the Sheffield Farms Company, Inc., New York, for generous supplies of materials used in the diets described in this paper.

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THE EFFECT OF PANTOTHENIC ACID DEFICIENCY UPON THE COENZYME A CONTENT AND PYRUVATE UTILIZATION OF RAT AND DUCK TISSUES*

BY ROBERT E. OLSON AND NATHAN O. KAPLAN

(From the Department of Nutrition, Harvard School of Public Health, the Biochemical Research Laboratory, Massachusetts General Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston)

(Received for publication, April 22, 1948)

Coenzyme A, which is essential for the acetylation of sulfanilamide in liver and acetylation of choline in brain, was found by Lipmann *et al.* (1) to be a pantothenic acid derivative. A ubiquitous distribution of this coenzyme in nature has been reported by Kaplan and Lipmann (2); it has also been observed, in general, that the coenzyme represents all of the pantothenic acid present in animal tissues (3). Novelli and Lipmann have shown* that *Proteus morganii*, *Lactobacillus arabinosus*, and yeast (4, 5) contain decreased amounts of coenzyme A when grown on pantothenic acid-deficient media and respond to added pantothenic acid by rapidly converting the vitamin to coenzyme A.

Dorfman, Berkman, and Koser (6) and Hills, (7) have shown that pantothenic acid is concerned with the oxidation of pyruvic acid in *Proteus morganii*. These findings were confirmed recently (4) and a correlation established between the stimulation of pyruvate oxidation after addition of pantothenic acid and the concurrent increases in coenzyme A content of the bacteria. Pilgrim, Axelrod, and Elvehjem (8) have reported that liver homogenates from pantothenic acid-deficient rats showed no increase in oxygen consumption with added pyruvate, while those from normal rats gave a marked response. These facts led us to study more closely the relationship between the concentration of tissue coenzyme A and pyruvate metabolism in representative avian and mammalian organisms.

This communication deals primarily with the effect of the dietary restriction of pantothenic acid upon the coenzyme A content and pyruvate metabolism of tissues from rats and ducks. Factors influencing the rate of coenzyme synthesis and destruction *in vitro* as well as the relative rates of coenzyme synthesis *in vivo* and *in vitro* were also studied.

* Supported in part by a grant-in-aid from the Life Insurance Medical Research Fund, New York, the Nutrition Foundation, Inc., New York, the Milbank Memorial Fund, New York, and in part (N. O. K.) by a grant from the Commonwealth Fund, New York.

EXPERIMENTAL

The animals and birds used in this study were 30 day-old male albino rats of the Hisaw strain and 7 day-old white Pekin ducklings. Twenty-four rats averaging 50 gm. in weight were divided into three groups and fed control, pantothenic acid-deficient, and riboflavin-deficient diets for 9 weeks. The control group received a normal diet of the following composition: casein 18 per cent, glucose 73 per cent, salts (9) 4 per cent, corn oil 3 per cent, cod liver oil 2 per cent; vitamin supplement containing the following quantities of B complex factors in crystalline form: thiamine 400 γ per 100 gm., riboflavin 800 γ , pyridoxine 400 γ , niacin 2000 γ , calcium pantothenate 2000 γ , choline chloride 100 mg. The two experimental groups were fed the same diet minus the riboflavin supplement and the pantothenic acid supplement respectively. Riboflavin deficiency was induced in order to evaluate the specificity of changes associated with pantothenic acid deficiency. After 3, 5, and 9 weeks on these diets animals were fasted 24 hours and sacrificed in pairs and the coenzyme A content of heart, liver, kidney, and adrenal determined by the procedure of Kaplan and Lipmann (2), which is based upon the enzymatic acetylation of sulfanilamide by aged extracts of pigeon liver. A unit of coenzyme A has been defined previously (2) and has been found to contain about 0.7 γ of pantothenic acid. At the same time, samples of heart and liver tissue were chilled in iced, oxygenated phosphate-saline of the following composition: NaCl 0.119 M, KCl 0.004 M, MgCl₂ 0.0005 M, Na₂HPO₄-NaH₂PO₄ 0.020 M, pH 7.4. After a few moments the tissues were sliced and incubated with and without added sodium pyruvate (5 mM per liter) in standard Warburg flasks for 2 hours. The oxygen consumption and total pyruvate utilization were determined. The details of these methods are presented elsewhere (Olson *et al.* (10)). The adrenal glands were fixed in formalin and frozen sections were stained by methods used to characterize ketosteroids (11). These were then kindly examined for signs of adrenal cortical exhaustion by Dr. Helen Deane of the Department of Anatomy.

The ducklings used in this study were received from the hatchery at 1 to 3 days of age and maintained in heated, raised bottom cages until they were 7 days of age, at which time they averaged 150 gm. in weight. They were then divided into experimental and control groups and fed their respective diets until loss of weight and a scaly dermatitis developed in the experimental group. The experimental diet consisted of casein 18 per cent, gelatin 10 per cent, Cellu flour 3 per cent, dextrose 50 per cent, corn oil 10.2 per cent, cod liver oil 2.0 per cent, salts (9) 5 per cent, CaHPO₄ 1.0 per cent, choline chloride 0.3 per cent, α -tocopherol in corn oil (10 mg. per ml.) 0.5 per cent. Supplementary crystalline vitamins were added in the following quantities per 100 gm. of diet: menadione 100 γ , thiamine 400

γ , riboflavin 800 γ , pyridoxine 400 γ , nicotinic acid 4 mg., biotin 20 γ , folic acid 100 γ . The control groups received 2.0 mg. of calcium pantothenate per 100 gm. of ration in addition. Acute deficiency signs developed in from 10 to 15 days. In one series of deficient ducklings, the birds were killed at 5 day intervals for 15 days and the coenzyme A content determined in liver and heart as a function of time. In another series, the oxygen consumption and pyruvate utilization of liver slices from deficient birds were determined by the standard Warburg technique in flasks with and without added pyruvate and with and without added sodium pantothenate at a level of 0.2 to 0.3 mm per liter. The pyruvate was always added at zero time from the side arm in an amount to make the final concentration 5 mm per liter. The period in these experiments was 1 hour, and the gas phase was O_2 . Both lactate production and pyruvate utilization were determined and the results expressed in terms of Q (microliters of metabolite produced or lost per mg. of dry weight of tissue per hour). In the experiments in which sodium pantothenate was added *in vitro*, the vitamin was added directly to the main chamber and allowed to react with tissue components during the period of equilibration. In the experiments in which pantothenate was added *in vivo* the dose was 10 mg. of sodium pantothenate per 100 gm. body weight, intraperitoneally, a dose calculated to give the same concentration of pantothenate per ml. of body water as was present in the tests *in vitro*. The deficient ducks injected with pantothenate were killed after 1 to 2 hours.

In the tests of factors influencing coenzyme A synthesis *in vitro*, livers from deficient ducklings were removed, chilled in oxygenated phosphate-saline, and sliced. The slices were then added to 50 ml. Erlenmeyer flasks and shaken at 37°. Coenzyme A was determined on the slices at the end of each experiment and occasionally in the medium.

Results

Coenzyme A and Pyruvate Utilization—The results of the first experiment with rats are shown in Figs. 1 and 2 and in Table I. Fig. 1 presents the mean growth of normal, riboflavin-deficient, and pantothenic acid-deficient rats plotted against the time in days. Each point on these growth curves represents the mean of from two to eight animals, depending upon the number remaining at the time. Deaths of rats in the experimental groups, apart from those caused experimentally at the 3, 5, and 9 week intervals, are indicated by small crosses in Fig. 1. In Fig. 2 each value plotted for coenzyme A represents a determination of the pooled tissues from two rats. It may be seen that the pantothenic acid-deficient rats showed marked decreases in coenzyme A content in all tissues examined, the absolute decreases being most prominent in the liver and adrenal. The

riboflavin-deficient animals showed coenzyme values not significantly different from normal on a unit weight basis despite the fact that they grew more poorly than the pantothenic acid-deficient group. The rise in the coenzyme A content of the livers of the normal and riboflavin-deficient rats after 3 weeks on the synthetic diets is probably due to the higher content of pantothenic acid of these experimental diets over that of the stock food. In heart, liver, and kidney there was a lag of 3 weeks before significant reduction in coenzyme A content occurred; in adrenal, it ap-

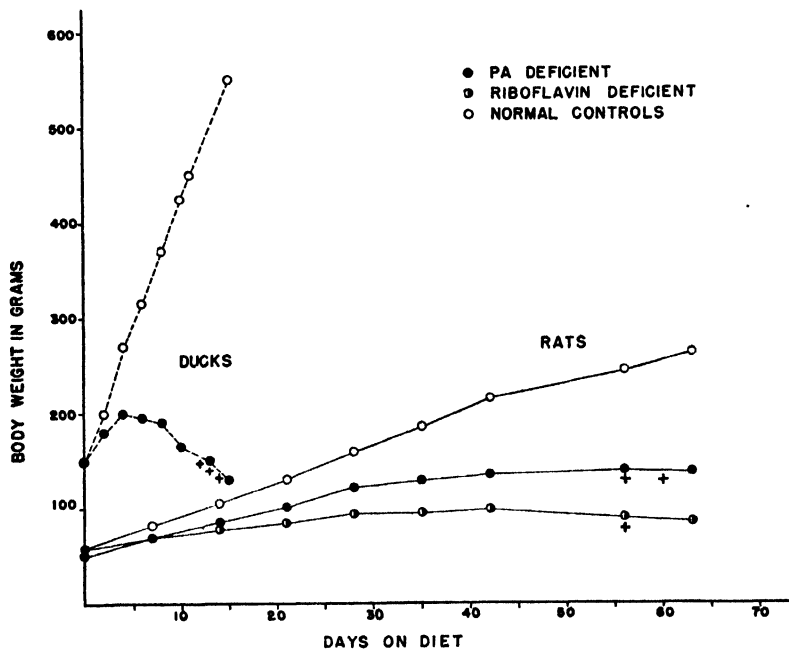


FIG. 1. Growth curves for normal and pantothenic acid-deficient ducks and normal, riboflavin-deficient, and pantothenic acid-deficient rats. Body weight in gm. is plotted against the time in days. All animals were fed *ad libitum*. The dotted lines are curves for ducks; the solid lines for rats. The crosses symbolize death. Each point represents results from two to eight animals.

peared to be immediate. Histological examination of the adrenals indicated that the same course of changes previously reported (11) in pantothenic acid deficiency in rats was found to occur in this series. Complete elimination of the lipides of the fasciculata occurred at the 9th week. Table I presents the preliminary findings on the oxygen consumption and pyruvate utilization of heart and liver slices taken from these rats at the same time as the tissues sampled for coenzyme A determination. There were no significant changes from normal in either the Q_{O_2} or the $-Q_{pyruvate}$

of either deficient group until after the 5th week on the deficiency diets. For this reason, the values obtained at the 3rd and 5th weeks are averaged

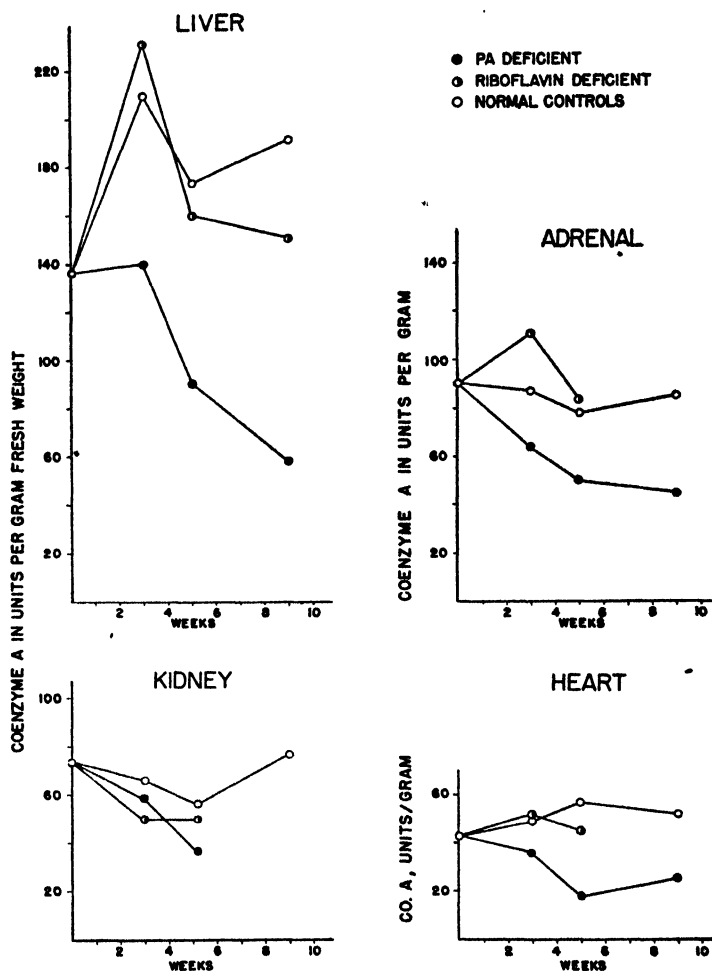


FIG. 2. Changes in the coenzyme A content of liver, adrenal, heart, and kidney in rats on normal, riboflavin-deficient, and pantothenic acid-deficient diets. Coenzyme A content in units per gm. of fresh weight of tissue plotted against the time on the diet in weeks. Each point represents the mean value for pooled tissues from two rats.

and compared with those obtained at the 9th week. All of the determinations on the controls are averaged. It may be seen that both heart and liver slices from pantothenic acid-deficient rats at the 9th week metabolized

pyruvate more poorly than did those from the controls or riboflavin-deficient rats. This was true despite the fact that the total oxygen consumption was reduced to a considerably greater extent in the riboflavin-deficient animals. These preliminary findings of a reduced pyruvate utilization in tissues from pantothenic acid-deficient rats were in good agreement with the report of Pilgrim, Axelrod, and Elvehjem (8) that pyruvate failed to stimulate oxygen uptake in homogenates of liver from pantothenic acid-deficient rats. The fact that this reduced pyruvate utilization occurred first when the tissues had become depleted of coenzyme A was of great interest and prompted further experimentation.

The peculiar sensitivity of young ducklings to dietary restriction of B complex vitamins (12) suggested to us that this bird might be a more

TABLE I

Effect of Pantothenic Acid and Riboflavin Deficiencies upon Oxygen Consumption and Pyruvate Utilization of Heart and Liver Slices in Fasted Rats

Group	Wks on diet	Heart tissue			Liver tissue		
		CO ₂ , no substrate	CO ₂ , pyruvate, 5 mm per liter	-Q _{pyruvate}	CO ₂ , no substrate	CO ₂ , pyruvate, 5 mm per liter	-Q _{pyruvate}
Control	3-9	8.2 ± 0.7*	10.4 ± 0.5	5.0 ± 0.3	6.6 ± 0.2	8.1 ± 0.2	4.5 ± 0.3
Riboflavin-deficient	3-5	8.2 ± 0.5	8.7 ± 0.2	5.6 ± 0.1	6.7 ± 0.2	8.2 ± 0.8	4.7 ± 0.9
	9	3.7 ± 0.1	5.1 ± 0.8	4.7 ± 0.8	5.8 ± 0.2	6.6 ± 0.2	3.5 ± 0.4
Pantothenic acid-deficient	3-5	7.1 ± 0.8	8.7 ± 0.1	4.8 ± 0.2	7.1 ± 0.1	9.0 ± 0.3	4.2 ± 0.2
	9	5.2 ± 0.2	6.0 ± 0.2	3.1 ± 0.2	6.8 ± 0.1	7.8 ± 0.4	1.7 ± 0.3

* The deviations are the standard error of the mean.

suitable experimental subject than the rat for the induction of pantothenic acid deficiency and the study of its relationship to pyruvate metabolism. Figs. 1 and 2 show the mean growth and mean coenzyme A content of heart and liver for deficient and normal birds. While the period for depletion of coenzyme A in rats appeared to be from 5 to 9 weeks in various tissues, depletion in the duckling took only 5 days (Fig. 3), at which time the coenzyme A concentration reached a low level which was not appreciably lowered until death. Table II shows that the rate of pyruvate utilization is markedly depressed in liver slices from such deficient ducks. The addition of sodium pantothenate to the Warburg flask *in vitro* during a 15 minute incubation period before addition to substrate did not appreciably improve the pyruvate utilization. The intraperitoneal injection of deficient birds with sodium pantothenate (10 mg. per

100 gm. of body weight), however, had a marked effect in restoring pyruvate utilization towards normal. This improvement in pyruvate utilization was accompanied by a rise in the coenzyme A content of the liver. In these experiments, the amount of pyruvate reduced to lactate was determined and the $-Q_{\text{pyruvate}}$ values corrected for lactate formation. The

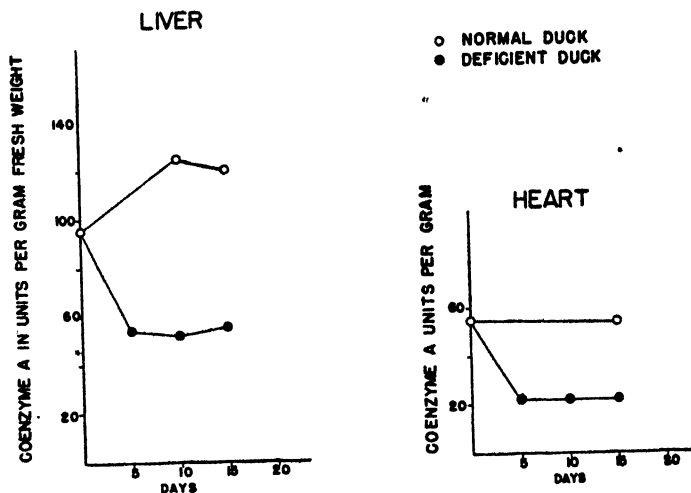


FIG. 3. Changes in the coenzyme A content of heart and liver from normal and pantothenic acid-deficient ducks. Coenzyme A in units per gm. of fresh weight is plotted against the time on the diet in days. Each point represents the mean value for pooled tissues for two ducks.

TABLE II
Effect of Pantothenic Acid Deficiency and Added Pantothenate upon Oxygen Consumption and Pyruvate Utilization of Duck Liver Slices

Group	No. of determinations	Coenzyme A, units per gm. fresh weight	QO_2 , no substrate	QO_2 , pyruvate 5 mM per liter	Net $-Q_{\text{pyruvate}}$
Deficient	4	45 ± 2	6.5 ± 0.2	7.3 ± 0.6	2.3 ± 0.7
“ treated <i>in vitro</i>	4	55 ± 3		8.7 ± 0.7	3.0 ± 0.4
“ “ <i>vivo</i>	6	117 ± 5	7.2 ± 0.8	9.2 ± 0.5	6.3 ± 0.6
Normal controls	6	130 ± 6	7.7 ± 0.5	9.8 ± 0.5	7.6 ± 0.5

net $-Q_{\text{pyruvate}}$ values thus obtained then serve as an index of the conversion of pyruvate into non-lactate products (10). It may be seen from Table II that the net $-Q_{\text{pyruvate}}$ of liver slices from deficient, treated, and normal ducks appears to be a more direct function of the coenzyme A content of the slice than the rate of oxygen consumption. In Fig. 4 the in-

dividual net $-Q_{\text{pyruvate}}$ values are plotted against their respective coenzyme A concentrations for liver slices from deficient, normal, and treated ducks and a strict proportionality between pyruvate utilization and coenzyme A concentration is observed.

In the ducks injected with pantothenate approximately 3 per cent of the injected vitamin was converted to coenzyme A in the liver. Two injections, or an interval longer than 90 minutes after injection, were not neces-

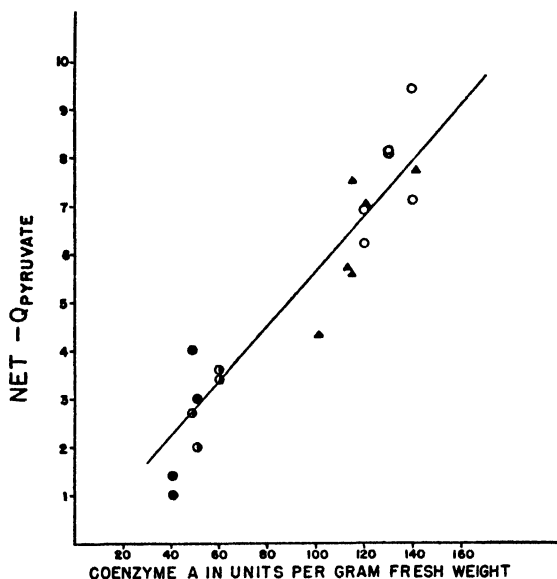


FIG. 4. Relationship between net pyruvate utilization (net $-Q_{\text{pyruvate}}$) and coenzyme A content of liver slices from deficient, pantothenic acid-treated, and normal ducks. Net $-Q_{\text{pyruvate}}$ values for individual liver slices are plotted against their respective coenzyme A values in units per gm. of fresh weight of slice. The following symbols represent the various groups: deficient (●), deficient treated *in vitro* (◐), deficient treated *in vivo* by intraperitoneal injection of 10 mg. of calcium pantothenate per 100 gm. of body weight 1 to 2 hours before observation (▲), and normal controls fed *ad libitum* (○).

sary for a maximum response. A summary of these data is given in Table III.

Synthesis of Coenzyme A in Tissue Slices from Deficient Ducks—The ability of tissue slices from deficient ducks to synthesize coenzyme A from added pantothenate *in vitro* was tested in a number of experiments which are summarized in Table IV. Although the increases in coenzyme A were relatively small when compared with the effect of pantothenate injected *in vivo*, the effect of the added vitamin is definite *in vitro* and indi-

cates that the surviving deficient tissue slice is now able to give rise to additional coenzyme from its precursors. There was no coenzyme found in the medium in these experiments. Both heart and liver slices demonstrated this property. Added pyruvate seemed to have no stimulatory effect upon coenzyme A synthesis, while added glucose appeared to inhibit the synthesis. The initial level of coenzyme A is well maintained in slices shaken in O₂ from 1 to 2 hours, slices from both deficient and normal ducks maintaining from 80 to 90 per cent of their original coenzyme A content under these conditions. Fig. 5 shows the relationship between the period of incubation and the coenzyme A content of liver slices from normal and

TABLE III

Effect of Intraperitoneal Injections of Sodium Pantothenate on Coenzyme A Content of Livers of Pantothenic Acid-Deficient Ducklings*

Experiment No.	Duck No.	No. of injections of sodium pantothenate	Time from 1st injection to sacrifice	Liver, fresh weight	Coenzyme A	
					Units per gm. wet weight	Units per liver
			min.	gm.		
1	1	0	0	6.8	78	525
	2	1	120	7.3	141	1003
	3	1	120	6.1	121	740
2	4	0	0	8.7	39	340
	5	1	90	6.0	101	606
	6	1	90	9.2	142	1312
3	7	0	0	10.1	28	285
	8	0	0	8.3	35	280
	9	1	120	7.5	118	890
	10	1	120	6.3	70	445
	11	2	240	8.4	115	970
	12	2	240	8.5	113	960

* The solution injected into the ducks was made up in (isotonic) phosphate-saline and contained 5 mg. of sodium pantothenate per ml. 10 mg. per 100 gm. of duck were given intraperitoneally.

deficient ducks incubated with and without added pantothenate at a level of 95 γ (0.2 mm per liter) of calcium pantothenate per ml. Synthesis of coenzyme A occurred in the deficient but not in the normal slices.

The effect of a number of additional variables was tested in a series of experiments summarized in Table V. It was found that adenylic acid when added together with pantothenic acid had no effect upon coenzyme synthesis. The addition of coenzyme A caused only little, if any, increase in the tissue concentration and was nearly quantitatively recovered from the medium after the period of incubation. The effect of fission products produced by enzymatic treatment of the coenzyme was also tested. Both

TABLE IV

Synthesis of Coenzyme A in Vitro from Added Pantothenate in Deficient Tissue Slices of Duck

All experiments conducted in phosphate-saline (PO_4 , 20 mm per liter) at 37.2° with O_2 as gas phase. All tissue slices from pantothenic acid-deficient ducks showing gross lesions and other signs of deficiency disease. Pyruvate, when added, was at 5 mm per liter, and glucose was at 11.1 mm per liter.

Experiment No.	Tissue slices	Period	Substrate added	Pantothenate, 0.2 mm per liter	Coenzyme A		
					Units per gm.	Increase,* units per gm.	Per cent increase
		<i>min.</i>					
1	Heart	60	None	0	23		
	"	60	"	+	35	12	52
	"	60	Pyruvate	+	34	11	48
2	"	60	None	0	19		
	"	60	"	+	25	6	32
	"	60	Pyruvate	+	25	6	32
3	Liver	0			49		
	"	60	Pyruvate	0	44	-5	-10
	"	60	"	+	63	19	43
4	"	0			52		
	"	60	Pyruvate	0	43	-9	-17
	"	60	"	0	50	-2	-4
	"	60	"	+	56	12	26
	"	60	"	+	61	15	32
5	"	0			58		
	"	60	Pyruvate	0	48	-10	-17
	"	60	"	0	50	-8	-12
	"	60	"	+	57	7	14
	"	60	"	+	65	15	30
6	"	0			52		
	"	0			50		
	"	60	None	0	47	-4	-8
	"	60	"	0	49	-2	-4
	"	60	Glucose	+	40	-8	-16
	"	60	"	+	44	-4	-8
	"	60	Pyruvate	+	60	12	25
	"	60	"	+	58	10	21

* Increases in coenzyme A in slices with added pantothenate were calculated with respect to the mean value obtained on the control slices incubated for the same period without added pantothenate; decreases in control slices were calculated with respect to the content of the unincubated slice.

alkaline intestinal phosphatase and a liver enzyme have been found¹ to be required for the liberation of pantothenic acid from coenzyme A.

¹ Novelli, G. D., Kaplan, N. O., and Lipmann, F., in preparation.

Dephosphorylated coenzyme seems to have less effect upon coenzyme A synthesis in liver tissues from deficient ducks than has pantothenic acid, as is seen in Experiment 5, Table IV. Treatment of coenzyme A with both the alkaline phosphatase and the liver enzyme, however, yields a product

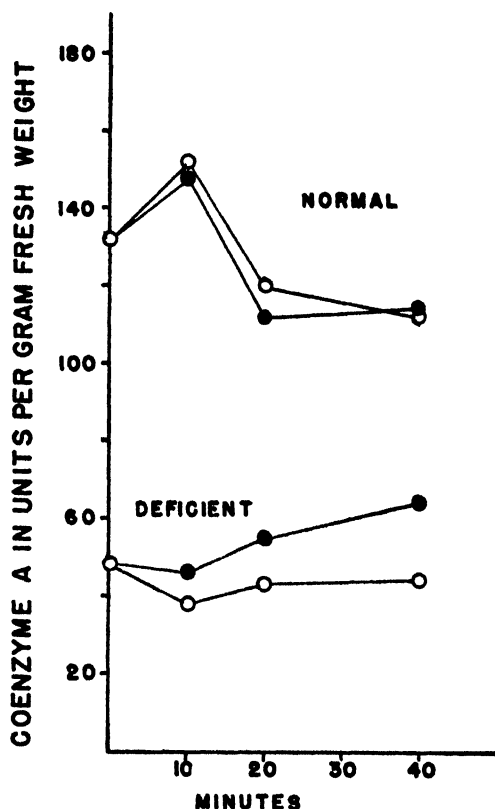


FIG. 5. Effect of incubating liver slices from normal and pantothenic acid-deficient ducks with added sodium pantothenate for 40 minutes. Coenzyme A in units per gm. of fresh weight of slice is plotted against the time in minutes. Slices were incubated at 37° in phosphate-saline in an atmosphere of O_2 . The upper pair of lines is from normal ducks; the lower pair from deficient ducks. Values obtained without added pantothenate (○), with added pantothenate (●).

which is similar to pantothenic acid in stimulating coenzyme A synthesis in the liver slice (Experiment 6, Table IV).

When liver slices from deficient ducks are incubated with added pantothenate in an atmosphere of N_2 , a marked fall in coenzyme A concentration occurs. Arsenite also depresses the level of coenzyme A in these slices.

DISCUSSION

A lowered pantothenic acid content of tissues from chicks fed a diet low in pantothenic acid was first reported by Snell, Pennington, and Williams (13). In this and a subsequent report (14) these workers observed decreases in the pantothenic acid content of tissues from deficient ducks of 50 per cent or more. Since it has been shown that substantially all of the pantothenic acid found in animal tissues is bound as coenzyme A (2), it would be reasonable to expect changes of this magnitude in the coenzyme A content of the tissues of pantothenic acid-deficient animals.

TABLE V

Effect of Various Factors and Coenzyme A Fission Products upon Coenzyme A Synthesis in Vitro in Deficient Duck Liver Slices

Medium, phosphate-saline; substrate, pyruvate, 5 mm per liter; time, 60 minutes; temperature, 37.2°.

Experiment No	No of determinations	Gas phase	Pantothenate, 0.3 mm per liter	Additional supplement	Coenzyme A, units per gm wet weight
1	4	O ₂	0		48 ± 2*
2	4	"	+		60 ± 2
3	2	"	+	Muscle adenylic acid, 3 mg. per flask	59 ± 5
4	4	"	0	Coenzyme A, 60 units per flask	54 ± 2
5	2	"	0	Alkaline phosphatase treated coenzyme A ≈ 60 units per flask	55 ± 0
6	2	"	0	Alkaline phosphatase-treated, liver-enzyme-treated coenzyme A ≈ 60 units per flask	63 ± 3
7	2	N ₂	+	0	15 ± 4
8	1	O ₂	+	Arsenite, 0.01 M	21

* The deviations are the standard error of the mean.

Decreases of 50 to 70 per cent in the tissues of deficient rats and ducks were found in this study.

Associated with this decrease in the coenzyme A content of heart and liver in both species there is a decrease in the ability of these tissues to metabolize pyruvic acid. The conversion of pyruvate to products other than lactate is sharply curtailed in liver slices from deficient ducks and is restored to approximately normal by the injection of pantothenic acid 1 to 2 hours before sacrifice of the birds. The injection of pantothenate also restores the coenzyme A levels to normal. The addition of pantothenate *in vitro* to liver slices from deficient ducks has a barely significant effect upon coenzyme A content and an insignificant effect upon pyruvate utilization. The changes in the oxygen consumption of heart and liver slices

from deficient rats and ducks incubated with added pyruvate are not as marked as the changes in total pyruvate utilization. These findings of changes in pyruvate metabolism of tissues from pantothenate-deficient rats and ducks confirm the earlier observations of Pilgrim, Axelrod, and Elvehjem (8, 15) with homogenates of liver from deficient rats.

It is known that coenzyme A is essential for the acetylation of aromatic amines in pigeon liver extracts (16) and Riggs and Hegsted (17) have recently found that rats placed upon pantothenic acid-deficient diets show a decreased ability to acetylate injected *p*-aminobenzoic acid as determined by measurement of acetyl-*p*-aminobenzoic acid in the urine. Decreases in the proportion of excreted *p*-aminobenzoic acid acetylated were found as early as 2 weeks after feeding the deficient diet, at about the time the first detectable decreases in the coenzyme A content of liver occur and long before frank deficiency signs were manifest. It would appear, therefore, that the acetylation of aromatic amines is more sensitive to decreases in the tissue content of coenzyme A than are other reactions such as those concerned in the catabolism of pyruvate.

Species differences in the sensitivity to dietary restriction of pantothenic acid are evident in a comparison of the time required to deplete the tissues of the rat and duck of their coenzyme A contents. In the rat, nearly 6 weeks are required to attain levels which appear to be minimal and resist further reduction; in the duck, the period is 5 days. The reason for the plateau in the coenzyme level after the initial drop is not completely clear. It might conceivably be due to a combination of coenzyme-protein complexes of varying stability, the most strongly bound remaining during the period of the plateau, or possibly to the establishment of a final steady state of coenzyme A synthesis and destruction due to minimal intestinal synthesis of pantothenic acid.

The rapid synthesis of coenzyme A after administration of pantothenic acid to deficient ducks resembles the conversion of pantothenic acid to coenzyme A in deficient yeast and bacteria (4, 5). The relatively small increase obtained with surviving tissue *in vitro* with added pantothenate is evidence for a synthetic system rigidly dependent upon the conditions of the living organism for its optimal operation. It is possible that supplementation of the slice with additional unknown reactants needed in the manufacture of the coenzyme would increase the yield.

Dorfman, Berkman, and Koser (6) and Hills (7) have suggested that the action of pantothenate in the oxidation of pyruvate by *Proteus morganii* is chiefly concerned with the acetate phase. The recent studies of Novelli and Lipmann (5) on pantothenic acid-deficient yeast indicate more directly that coenzyme A is intimately associated with the metabolism of acetate. The acetylation of choline in brain, the acetylation of sulfanilamide in

liver, and the formation of acethydroxamic acid from adenosine triphosphate and acetate (18, 19) are all reactions which require coenzyme A and strengthen the inference that coenzyme A is closely linked with acetate metabolism.

Dorfman, Berkman, and Koser (6) also find that pantothenic acid will stimulate pyruvate oxidation in *Proteus morganii* even when acetate is the end-product. Our results would support equally well the possibility that the conversion of pyruvate to active acetate is a reaction catalyzed in part by coenzyme A. Such a possibility is now under investigation.

SUMMARY

Rats maintained on pantothenic acid-free diets for periods up to 9 weeks maintain normal coenzyme A content in their tissues for 2 to 3 weeks and then show a gradual depletion to a level 35 to 40 per cent of normal.

Ducklings show a more rapid depletion of coenzyme A in their tissues, to 40 per cent of normal, when placed upon a pantothenic acid-deficient diet.

Liver slices from rats and ducks deficient in pantothenic acid show a decreased ability to utilize pyruvate. This accompanies a low coenzyme A content.

Injection of pantothenate into deficient ducks results in a large increase in coenzyme A in liver and increases the ability of surviving liver slices to utilize pyruvate.

Addition of pantothenate to heart and liver slices from deficient ducks results in small but definite increases of coenzyme A, of the order of 30 per cent. Normal slices do not synthesize additional coenzyme A under these conditions.

Added coenzyme A and its cleavage products do not increase the coenzyme A synthesis in deficient tissue slices any more than their respective free pantothenic acid equivalents.

Nitrogen, arsenite, and glucose interfere with the synthesis of coenzyme A *in vitro*.

The authors are greatly indebted to Dr. Fritz Lipmann for his constant stimulation and helpful suggestions, both during the conduct of the study and the preparation of the manuscript. We are also indebted to Miss Charlotte Thrall for technical assistance in the performance of these experiments.

We wish to thank Merck and Company, Inc., Rahway, New Jersey, the Corn Industries Research Foundation, New York, and the Sheffield Farms

Company, Inc., New York, for generous supplies of materials used in these diets.

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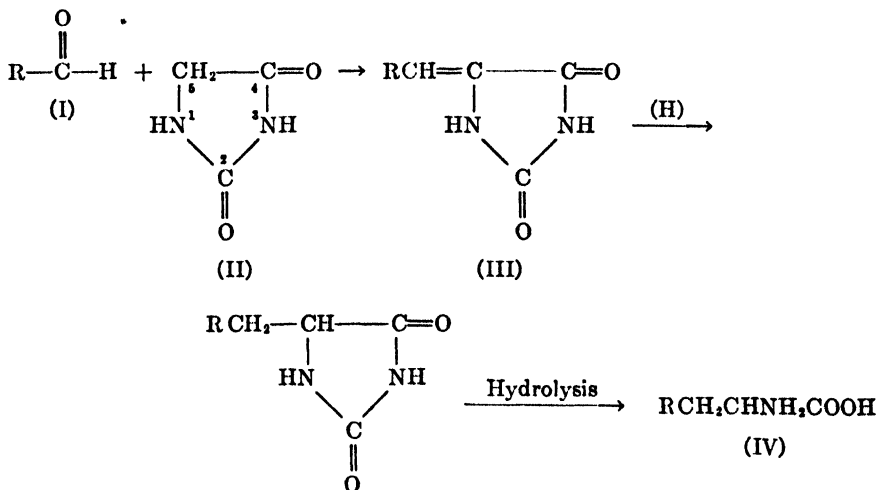
SYNTHESIS OF CARBOXYL-LABELED TRYPTOPHAN FROM HYDANTOIN CONTAINING ISOTOPIC CARBON

By HOWARD W. BOND

(From the Experimental Biology and Medicine Institute, National Institute of Health,
Bethesda, Maryland)

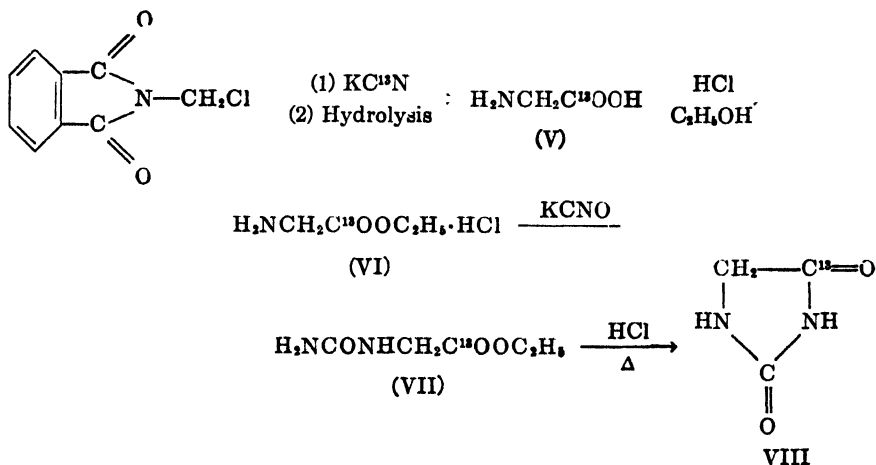
(Received for publication, May 8, 1948)

One of the general methods for the preparation of α -amino acids is based on the reaction of the appropriate aldehyde (I) with hydantoin (II), giving the aldehyde hydantoin (III), which is first reduced and then hydrolyzed, giving the corresponding α -amino acid (IV). The preparations by this procedure of phenylalanine (1, 2), tyrosine (1), and tryptophan (3, 4) have been described.



The use of hydantoin labeled with isotopic carbon in position 4 in the synthesis would lead to the corresponding carboxyl-labeled amino acid. This has been demonstrated by the preparation of labeled hydantoin and its conversion to carboxyl-labeled tryptophan.

Hydantoin-4- C^{14} was prepared in four steps from isotopic potassium cyanide according to the scheme, potassium cyanide $\rightarrow$ glycine (V) $\rightarrow$ glycine ethyl ester hydrochloride (VI) $\rightarrow$ hydantoic ethyl ester (VII) $\rightarrow$ hydantoin (VIII).



The yield of hydantoin based on potassium cyanide was 57 per cent. The yield of tryptophan based on hydantoin was 59 per cent, giving an over-all yield from potassium cyanide of 33 per cent.

EXPERIMENTAL

Synthesis of Hydantoin Containing Isotopic Carbon

Isotopic Glycine—The directions of Sakami, Evans, and Gurin (5) were followed exactly. From 2.68 gm. of isotopic potassium cyanide which analysis¹ showed to contain 20.7 per cent excess C¹³, 1.93 gm. (62.5 per cent) of glycine, m.p. 236–237° (decomposition),² was obtained. An authentic sample of glycine melted at 235° (decomposition).

Isotopic Glycine Ethyl Ester Hydrochloride—The directions of Harries and Weiss (6) were followed. The labeled glycine was covered with 10 ml. of absolute ethanol and dry hydrogen chloride was bubbled through the mixture while suspended in a 65–70° oil bath. The glycine slowly dissolved during the course of 30 minutes. Alcohol was added to replace that which evaporated. Treatment with hydrogen chloride was continued for an additional 15 minutes. In practice runs the ester hydrochloride would occasionally crystallize during this last period. The mixture was cautiously evaporated to dryness on a warm surface, giving 3.49 gm. (98.0 per cent) of glycine ethyl ester hydrochloride, m.p. 145.2°.

Isotope Analysis—Excess C¹³. Calculated, 5.18; found, 5.46, 5.98

¹ All Analyses were performed on the consolidated mass spectrometer of the National Bureau of Standards. I wish to thank Dr. Fred L. Mohler and his staff for these analyses.

² All melting points were taken with the same apparatus, except where noted, and are uncorrected.

Isotopic Hydantoic Ethyl Ester—The directions of Harries and Weiss were followed. The labeled glycine ethyl ester hydrochloride was dissolved in 4 ml. of water, and to it was added a slurry of 3.0 gm. of freshly prepared potassium cyanate (7) in 7.0 ml. of water. The mixture was stirred until precipitation started (4 to 5 minutes), and was then cooled at -5° for 2 hours, filtered, and dried, giving 3.40 gm. (92.4 per cent) of hydantoic ethyl ester, m.p. $129.3-129.8^{\circ}$.

Isotope Analysis—Excess C^{13} . Calculated, 4.14; found, 4.21, 4.31

Isotopic Hydantoin—The directions of Harries and Weiss and of Wagner and Simons (8) were followed. The labeled hydantoic ethyl ester was covered with 15 ml. of 25 per cent hydrochloric acid and evaporated to dryness on a steam bath overnight, giving 2.67 gm. of crude hydantoin. This product was obviously impure, since the yield exceeded the theoretical (2.33 gm.) and the melting point, $203-206^{\circ}$, was low. Fortunately, however, it was found in trial runs that this crude material could be used directly for the synthesis of indolyldeneydantoin without recourse to purification by alcohol extraction such as that recommended by Wagner and Simons.

Since in trial runs in which purification was effected, the yield of hydantoin regularly approached quantitative amounts, such a yield is assumed here, giving an over-all yield of hydantoin based on potassium cyanide of 57 per cent.

Isotope Analysis—Excess C^{13} . Calculated, 6.91; found, 6.86, 6.97

Synthesis of Carboxyl-Labeled Tryptophan

Isotopic Indolyldeneydantoin—The directions of Shabica *et al.* (9) were followed. The labeled hydantoin was mixed with 3.38 gm. of indole-3-aldehyde and 9.0 ml. of piperidine in a 100 ml. flask equipped with a reflux condenser. The flask was lowered into an oil bath maintained at 150° for 20 minutes. The canary-yellow indolyldeneydantoin which formed was suspended in 250 ml. of water, acidified to Congo red paper with acetic acid, filtered, and washed with water, giving 5.00 gm. (94.5 per cent) of indolyldeneydantoin, m.p. $314-314.5^{\circ}$ (metal block); authentic sample, m.p. 314° .

Isotope Analysis—Excess C^{13} . Calculated, 1.73; found, 1.50, 1.62

Isotopic Indolylmethylhydantoin—The directions of Elks *et al.* (10) were followed exactly. It was found in practice that the Raney nickel used had to be quite active; otherwise the period of hydrogenation was unduly prolonged from the normal 6 hours to about 24, and the resulting product melted over a wide range after softening at 218° . Fortunately, however, this product gave tryptophan upon hydrolysis. It was presumed that

partial hydrolysis occurred during the long contact with sodium hydroxide.

The yield of indolylmethylhydantoin was 3.57 gm. (71.0 per cent), m.p. 218°. The yield in practice runs was usually about 90 per cent.

Isotope Analysis—Excess C¹³. Calculated, 1.73; found, 1.75, 1.76

Isotopic Tryptophan—The directions of Elks *et al.* were followed except for two modifications: the indolylmethylhydantoin was hydrolyzed for 40 instead of 24 hours, and the final product was evaporated to dryness on a steam bath instead of *in vacuo*. Yield, 2.80 gm. (88.0 per cent), m.p. 268°.

Isotope Analysis—Excess C¹³. Calculated, 1.88; found, 1.93, 1.98

The isotopic tryptophan and unlabeled tryptophan made by the same process in practice runs showed full tryptophan activity when tested by biological assay.³

The author is indebted to Mr. Clarence E. Emery, Jr., for technical assistance in carrying out many of the practice runs.

SUMMARY

Hydantoin labeled with isotopic carbon has been prepared from isotopic potassium cyanide in 57 per cent yield.

Carboxyl-labeled DL-tryptophan has been prepared from the labeled hydantoin in 59 per cent yield.

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³ Analysis by Dr. James M. Hundley of this Institute.

URINARY EXCRETION OF NICOTINIC ACID AND N¹-METHYLNICOTINAMIDE BY RATS FED TRYPTOPHAN AND DIETS DEFICIENT IN VARIOUS B VITAMINS*

By PLINIO B. JUNQUEIRA† AND B. S. SCHWEIGERT‡

(From the Department of Biochemistry and Nutrition, Agricultural and Mechanical
College of Texas, College Station)

(Received for publication, May 7, 1948)

The inability of vitamin B₆-deficient rats and mice to convert dietary tryptophan to nicotinic acid and its metabolites has been observed by several workers (1-4). Further, when vitamin B₆ supplements were given to rats previously deficient in the vitamin, the animals usually recovered the ability to transform tryptophan into nicotinic acid metabolites, although the response was apparently influenced by the severity of the deficiency of vitamin B₆ (2-4). Animals fed diets adequate in vitamin B₆ but differing in quality and quantity of protein or in the type of carbohydrate fed do, however, synthesize large amounts of nicotinic acid metabolites when tryptophan is fed (5-7).¹ Considerable speculation has arisen as to the site of conversion of tryptophan to nicotinic acid derivatives, particularly as to the rôle of intestinal microorganisms and of synthesis in the tissues of the animal. Recent findings have revealed that at least some of the synthesis of nicotinic acid from tryptophan can occur within the tissues (8) in studies conducted without the complicating effects of intestinal microorganisms.

This biochemical transformation has stimulated a great deal of interest, therefore, not only in the effect of dietary factors on the apparent conversion of tryptophan to nicotinic acid, but in the site of synthesis. In the present study, experiments were conducted to determine whether rats deficient in vitamins other than vitamin B₆ were able to convert tryptophan to nicotinic acid. The urinary excretion of nicotinic acid and its methylated derivative

* We wish to thank Helen Keene and Frances Panzer for valuable technical assistance.

Supported in part by a grant from Swift and Company and the National Livestock and Meat Board. We are also indebted to The Dow Chemical Company for generous supplies of DL-tryptophan, to Merck and Company, Inc., for the pyridoxine hydrochloride, nicotinic acid, biotin, and anthranilic acid, and to Sharp and Dohme for the succinylsulfathiazole used in these studies.

† Graduate fellow from São Paulo, Brazil, sponsored in part by the Institute of International Education.

‡ Present address, Division of Biochemistry and Nutrition, American Meat Institute Foundation, University of Chicago, Chicago.

¹ Schweigert, B. S., unpublished data.

was determined when rats were fed adequate diets, diets containing succinylsulfathiazole, or diets deficient in thiamine, riboflavin, pantothenic acid, vitamin B₆, pteroylglutamic acid (folic acid), or in calories. In addition, studies were conducted to determine the effects of feeding L- or DL-tryptophan and related compounds on the urinary excretion of nicotinic acid metabolites.

EXPERIMENTAL

Care of Animals—Unless indicated otherwise, weanling rats of the Sprague-Dawley strain were used. They were housed in single cages and four rats were used in each group in each experiment. After 3 weeks on the experiment two to four of the rats were selected for metabolism studies. The procedures used were similar to those employed in earlier work (1, 4). Quantitative urine collections were made simultaneously for all groups in each experimental series for three or more 2 to 3 day periods when the basal diets were fed, and three or more alternative periods when the basal diets plus tryptophan were fed. This sequence of experimentation was varied slightly for individual experiments. Food consumption and weight records were kept for all animals used in the metabolism studies.

Composition of Diets—The control diet used was composed of (in per cent) purified casein 24, sucrose 67, mineral mixture (9) 4, corn oil 4.7, vitamins A and D concentrate 0.3, and the following amounts of B vitamins per 100 gm.: thiamine 250 γ , riboflavin 300 γ , pyridoxine 250 γ , calcium pantothenate 2 mg., choline 100 mg., inositol 100 mg., biotin 10 γ , and synthetic pteroylglutamic acid (folic acid) 200 γ . The vitamin B-deficient diets were prepared by omitting the appropriate vitamin from the ration. The effect of feeding succinylsulfathiazole was tested with diets in which pteroylglutamic acid was omitted and with diets containing this vitamin. 1 per cent of the drug was added at the expense of the carbohydrate. Details of the dietary treatment are indicated for each experiment.

Results

Experiment 1. Effect of Feeding Succinylsulfathiazole and Diets Deficient in Pantothenic Acid, Thiamine, Vitamin B₆, or Riboflavin—In the first experiments the following dietary treatments were used: control diet previously described and the diets indicated by the vitamin omitted; pteroylglutamic acid (— PGA), pteroylglutamic acid plus succinylsulfathiazole (— PGA + SS), calcium pantothenate (— PA), thiamine (— B₁), pyridoxine (— B₆), and riboflavin (— flavin). The sequence of metabolism experiments previously indicated was used and the results obtained, either when the basal diets or the basal diets plus 100 mg. of DL-tryptophan per day were fed, are shown for the last two collection periods in Table I. The

gain per week and the average food consumption are shown and are tabulated for the 3rd to the 5th weeks on experiment, during which time these metabolism collections were made. N¹-Methylnicotinamide and nicotinic acid were determined by methods previously described (1).

It was necessary to supplement the thiamine- and riboflavin-deficient groups with small doses of the appropriate vitamin to prevent death during the 4th and 5th weeks. The tryptophan supplement was mixed with the daily allocation of food and the food consumption was restricted slightly to insure complete consumption of the tryptophan supplement.

TABLE I

Urinary Excretion of Nicotinic Acid and N¹-Methylnicotinamide by Rats Fed Diets with and without Added Tryptophan and Deficient in Various B Vitamins

Each rat received the respective basal diets indicated by (–) and the basal diets + 100 mg. of DL-tryptophan per day, indicated by (+).

Period No.		Dietary regimen*						
		+ or – tryptophan	Control diet	– PGA	– PGA + SS	– PA	– B ₁	– B ₂ flavin
	Gain per wk., gm Food consumption per day, gm.		32 10.6	39 12.0	8 9.2	4 6.5	–1 2.1	3 5.1
I	N ¹ -Methylnicotinamide excretion, γ per rat per day	–	313	384	104	252	134	46
		+	3817	2667	1218	1733	548	275
	Nicotinic acid excretion, γ per rat per day	–	28	24	13	21	11	7
II		+	375	325	83	147	25	13
	N ¹ -Methylnicotinamide excretion, γ per rat per day	–	950	613	314	500	247	110
		+	3075	2300	625	1424	441	259
	Nicotinic acid excretion, γ per rat per day	–	62	72	20	33	14	11
		+	225	371	25	108	13	15

* See the text for a complete description of the diets used.

It will be noted that large amounts of N¹-methylnicotinamide and nicotinic acid were excreted when tryptophan was fed to rats receiving the basal diet, or the basal diet devoid of added pteroylglutamic acid. The addition of succinylsulfathiazole resulted in a considerable reduction in the amounts excreted, particularly in Period II. Some reduction was noted when the pantothenic acid-deficient diet was fed. However, the excretion of nicotinic acid metabolites was much higher than that observed when either thiamine, riboflavin, or the vitamin B₆-deficient diet was fed. These findings indicated, therefore, that rats deficient in factors other than vitamin B₆ were also unable to convert dietary tryptophan effectively to nicotinic acid metabolites.

An inspection of these results indicated several factors that may be in-

volved. It can be seen that the magnitude of the values for nicotinic acid derivatives is correlated somewhat with the food intake. This is not specific, however, since the food consumption of the — PGA + SS group was higher than for the PA-deficient group and the excretion values were the reverse. Further, the food intake of the vitamin B₆-deficient group was higher than for the vitamin B₁- or riboflavin-deficient group and the nicotinic acid excretion was the lowest for the vitamin B₆-deficient group. Similar exceptions to correlations in rate of gain and urinary excretion data are readily apparent. Since the vitamins were added to the ration and the voluntary food intake varied for each group, the intake of the vitamins, *i.e.* vitamin B₆, which is known to affect the efficiency of this conversion, was necessarily different for the different groups. The preliminary effects noted when sulfasuxidine was fed were somewhat surprising and groups which received pteroylglutamic acid and sulfasuxidine were needed to clarify these observations.

Additional groups in this and later experiments were fed 1 mg. of nicotinic acid in addition to the control diet. The growth rates for these animals approximated those for the groups fed the control diet without nicotinic acid, thus substantiating earlier observations (6) that nicotinic acid supplements do not increase the rate of growth when 24 per cent casein is fed.

Experiment 2. Effect of Pteroylglutamic Acid in Counteracting Effect of Ingestion of Succinylsulfathiazole and Further Studies on Other Vitamin B Deficiencies—The results obtained in Experiment 1 were extended in a subsequent experiment which included the following groups: control diet fed *ad libitum*, control diet restricted to the food intake of the vitamin B₁-deficient groups, control plus SS, control plus SS and restricted food intake; the control diet with the following vitamins omitted: pteroylglutamic acid (— PGA), pteroylglutamic acid plus SS, calcium pantothenate (— PA), thiamine (— B₁), pyridoxine (— B₆), and riboflavin (— flavin). To eliminate variable consumption of the vitamins by different groups, the vitamin B supplements were given orally by dropper calibrated to provide one-tenth the amounts indicated in the section, "Composition of the diets," each day. The appropriate vitamin was omitted from the supplement and the solutions were refrigerated when not in use. Tryptophan supplements were provided in a manner similar to that used in Experiment 1, except that 50 mg. of L-tryptophan were fed per day. L-Tryptophan was used, since it was possible that the utilization of D-tryptophan from DL-tryptophan as a precursor of nicotinic acid may be reduced when the vitamin-deficient diets were fed. The results obtained for these studies are presented in Table II. These results are summarized for the 3rd to 5th weeks on the experiment, which includes three collection periods when the basal diet and three when tryptophan supplements were

fed. The values for N¹-methylnicotinamide only are included, since the relative values for nicotinic acid for this and other experiments are in good agreement with the data obtained for N¹-methylnicotinamide, the major excretory product in the rat.

Again it was necessary to supplement the vitamin B₁ and riboflavin-deficient groups during the latter phases of the experiment. Although restriction of the food intake by the control and control plus SS groups to that consumed by the vitamin B₁-deficient group was not completely successful, the food intake approximated that of the vitamin B₁-deficient group (4.6 and 4.2 gm. per day, respectively). It was clear that a reduced

TABLE II

Urinary Excretion of N¹-Methylnicotinamide by Rats Fed Diets with and without Additional Tryptophan and Deficient in Various B Vitamins or Calories

Each rat received the respective basal diets indicated by (–) and the basal diets + 50 mg. of L-tryptophan per day, indicated by (+).

Dietary regimen	Gain per wk.	Food con- sumption per day	N ¹ -Methylnicotinamide excretion, γ per rat per day (+ or – tryptophan)					
			Period I		Period II		Period III	
			–	+	–	+	–	+
Control, <i>ad libitum</i>	27	12.6	485	1916	429	1210	393	1175
“ restricted	–2	4.6	104	507	131	354	141	764
“ + SS	33	12.1	354	2290	742	1675	691	1630
“ + “ restricted	–2	4.6	127	575	254	559	219	471
– PGA	28	10.0	333	1885	468	1280	480	1415
– “ + SS	1	6.3	215	647	139	373	449	319
– PA	5	6.5	221	1015	275	1280	345	1280
– B ₁	–2	4.2	131	487	238	511	220	331
– B ₂	2.5	6.2	184	277	232	275	305	406
– flavin	3	4.3	144	312	193	400	291	594

food intake did markedly reduce the urinary excretion of N¹-methylnicotinamide. This was observed for both the groups receiving and not receiving succinylsulfathiazole. The restricted groups did, however, excrete somewhat more N¹-methylnicotinamide than did those receiving vitamin B₁-, B₂-, or riboflavin-deficient diets.

The excretion of N¹-methylnicotinamide was again reduced when succinylsulfathiazole was fed and no pteroylglutamic acid was added (–PGA + SS). This effect, however, was completely counteracted either by the addition of pteroylglutamic acid (control plus SS) or by the omission of the succinylsulfathiazole with no added pteroylglutamic acid (–PGA). The rôle of pteroylglutamic acid in counteracting the effect of feeding

succinylsulfathiazole was therefore established. The small increases noted when tryptophan was fed to vitamin B₁-, B₆-, or riboflavin-deficient rats, as compared to those when the basal diets were fed, were confirmed. Further, the excretion of N¹-methylnicotinamide observed when tryptophan was ingested by the pantothenic acid-deficient group approximated that of the control groups. These results were in agreement with those obtained in the first experiments and indicated that the methods of B vitamin supplementation were not complicating factors.

After the termination of these experiments, the vitamin B₆- and thiamine-deficient groups were supplemented with 25 γ per rat per day of the appropriate vitamin and, in successive periods of recovery when tryptophan

TABLE III

Urinary Excretion of Nicotinic Acid Metabolites after Ingestion of Tryptophan by Rats Fed Diets Deficient or Adequate in Vitamin B₆ and Vitamin B₁

The results are expressed in micrograms per rat per day.

Dietary regimen	Days on experiment	N ¹ -Methyl-nicotinamide	Nicotinic acid	N ¹ -Methyl-nicotinamide	Nicotinic acid
		Vitamin B ₆ -deficient group		Vitamin B ₁ -deficient group	
Basal diet	32-34	305	15.5	220	13.1
" + tryptophan	35-37	406	13.1	331	27.7
		+ 25 γ pyridoxine per day		+ 25 γ thiamine per day	
" diet	38-40	167	10.2	126	13.9
" + tryptophan*	41-43	1004	51.5	941	77.0
" diet	44-46	200	20.0	273	27.7
" + tryptophan*	47-49	2039	127	1165	139
" diet	50-51	435	34.7	315	45.7
" + tryptophan*	52-54	2410	174	2119	144

* 100 mg. of DL-tryptophan per rat per day.

was fed, the response was noted in the amounts of N¹-methylnicotinamide and nicotinic acid excreted. It will be noted that a rapid recovery in the ability of the animal to excrete the nicotinic acid derivatives occurred (Table III). The results obtained in the pretest period and after supplementation have been presented in Table III. DL-Tryptophan was fed in the recovery experiments in order to conform to the technique used in previous studies on the recovery from a vitamin B₆ deficiency (4). The recovery noted was not only rapid but similar for both the vitamin B₆- and thiamine-supplemented groups.

Experiment 3. Effect of Food Intake—It seemed desirable to obtain additional information on the effect of the amount of food ingested on the

apparent conversion of tryptophan to nicotinic acid metabolites. In Experiment 3 groups were fed 6, 8, or 10 gm. of the control diet. Other tests included were the control diet fed *ad libitum*, vitamin B₆-deficient, and riboflavin-deficient diets. The B vitamins were again provided daily by dropper, as previously described. The vitamin B₆- and riboflavin-deficient groups were selected in order to obtain further comparisons with

TABLE IV

Correlation of Excretion of Nicotinic Acid Metabolites with Tryptophan Ingestion, Body Weight, and Gain of Rats Fed Various Diets

Each figure is an average of the results obtained for four collection periods when the basal diets were fed and four periods when the basal diets + 100 mg. of L-tryptophan were fed. Simultaneous urine collections were made for two rats in each group during each collection period; consequently the values indicated are averages of eight determinations.

	+ or - tryptophan	Experimental regimen					
		- B ₆	- ribo- flavin	Complete, <i>ad libitum</i>	Complete, restricted		
Food intake per day, gm.		6.2	5.6	13.6	10	8	6
Rate of gain, gm. per wk.		3.0	3.0	29	16	9	6.7
Urinary excretion of N ¹ -meth- ylnicotinamide, γ per rat per day	+	509	543	3152	3223	1313	1277
	-	156	165	480	383	77	103
Urinary excretion of nicotinic acid, γ per rat per day	+	30	36	215	178	96	70
	-	15	16	40	28	19	15
Urinary excretion of N ¹ -meth- ylnicotinamide, γ per 100 gm. body weight	+	508	740	1576	2050	1050	1470
	-	155	220	240	244	62	118
Urinary excretion of nicotinic acid, γ per 100 gm. body weight	+	30	49	108	102	77	80
	-	15	22	20	18	15	17
Tryptophan ingestion, mg. per rat per day	+	114	113	135	126	121	115
	-	18.7	14.8	40.6	26.4	21.1	15.8
Urinary excretion of N ¹ -meth- ylnicotinamide, γ per mg. of ingested tryptophan	+	4.5	4.8	23.4	25.5	10.8	11.0
	-	8.3	11.1	11.8	14.5	3.7	8.7
Urinary excretion of nicotinic acid, γ per mg. of ingested tryptophan	+	0.26	0.32	1.59	1.41	0.79	0.61
	-	0.8	1.1	1.1	1.1	0.9	1.0

groups fed vitamin-deficient or adequate diets, but with comparable food intake. In Experiment 3 the tryptophan supplement was increased to 100 mg. of L-tryptophan per day in order to magnify the differences observed in the amount of nicotinic acid derivatives excreted in the urine by groups receiving different dietary treatments.

The results of this experiment are summarized in Table IV for four

collection periods made during the 3rd to 6th weeks. The excretion of the nicotinic acid metabolites when the control diets were fed is influenced by the caloric intake. The amount excreted was not reduced when 10 gm. were fed per day, but was considerably reduced when only 8 or 6 gm. were fed per day. The amount excreted per day by the latter two groups was at least twice that excreted by the vitamin B₆- or riboflavin-deficient groups, even though the food intake of the vitamin-deficient groups approximated 6 gm. per day.

The excretion of nicotinic acid metabolites by the various groups in different experiments has also been compared on a body weight basis, on the basis of tryptophan ingestion, and per gm. of food consumed. The results obtained for the different groups included in Experiment 3 are indicated in Table IV for some of these comparisons. It will be noted that, regardless of whether the results are expressed on the basis of the amount excreted per day, per 100 gm. of body weight, per mg. of tryptophan consumed, or per gm. of food consumed (1), a several fold increase in the effectiveness of the conversion of tryptophan to nicotinic acid was obtained when the control diets were fed, as compared to the increase when the vitamin-deficient diets were fed. The tryptophan content of the casein in the diet was determined microbiologically (10). The amount of tryptophan excreted as nicotinic acid metabolites when the control diet was fed averaged 2 to 4 per cent of the intake.

It may be concluded, therefore, from these experiments that the urinary excretion of nicotinic acid metabolites when tryptophan is fed is relatively insensitive to the inclusion of succinylsulfathiazole in diets containing pteroylglutamic acid, to the omission of pteroylglutamic acid from the diets, or to the omission of pantothenic acid. On the other hand, a marked reduction in the urinary excretion of nicotinic acid derivatives occurs when succinylsulfathiazole is added to diets not containing pteroylglutamic acid, when vitamin B₆, thiamine, or riboflavin is omitted from the diet, or when the caloric intake is severely restricted.

Miscellaneous Studies—It was of interest to test the comparative effectiveness of L- and DL-tryptophan and also of compounds related to tryptophan such as anthranilic acid as nicotinic acid precursors. In these studies young, mature male rats were used (200 to 250 gm. in weight) and the diet was the control diet used in the other studies. In most cases the same rats were used during different collection periods when L- and DL-tryptophan or anthranilic acid was fed. Three or more rats were used in each group and the results are presented in Table V. D-Tryptophan was shown to be active as a precursor of nicotinic acid in each of the tests conducted. It may be concluded from these tests that D-tryptophan is utilized essentially as effectively as L-tryptophan, while the ingestion of anthranilic acid

resulted in a rise in the excretion of N¹-methylnicotinamide but not in nicotinic acid. The results with anthranilic acid are, therefore, not conclusive. Tests conducted with anthranilic acid revealed that it did not interfere with the determination of N¹-methylnicotinamide. The response noted, therefore, may have been due to a metabolite of anthranilic acid which was measured as N¹-methylnicotinamide or to the production of N¹-methylnicotinamide. Similar findings were noted when indole was

TABLE V*

Effect of Ingestion of L-Tryptophan, DL-Tryptophan, or Anthranilic Acid on Excretion of N¹-Methylnicotinamide and Nicotinic Acid by Rat

The results are expressed in micrograms per rat per day.

Group No.	Dietary regimen	N ¹ -Methyl-nicotin- amide	Nicotinic acid	N ¹ -Methyl-nicotin- amide	Nicotinic acid
		L-Tryptophan, 50 mg. per rat per day		DL-Tryptophan, 100 mg per rat per day	
I	Basal diet	953	50	779	50
	" + tryptophan	1983	113	3890	131
II	" diet	1403	75	929	66
	" + tryptophan	2643	117	3217	184
III	" diet	240	23	218	35
	" + tryptophan	813	43	1212	61
III-A	" diet	220	24		
	" + tryptophan*	1269	44		
Anthranilic acid					
I	" diet	587	60.6		
	" + anthranilic acid	1464	43.2		
II	" diet	342	34.6	646	46.7
	" + anthranilic acid	1134	43.5	1795	42.9

* Group III-A received 100 mg. per rat per day.

fed (11); however, in this case it was demonstrated that indole itself interfered with the determination of N¹-methylnicotinamide.

A summary of the results obtained, when tryptophan and related compounds were fed, on the urinary excretion of nicotinic acid and N¹-methylnicotinamide shows that compounds related to tryptophan that have been tested are either inactive or much less active than L- or D-tryptophan. Many investigators have shown that L-tryptophan is active as a precursor of nicotinic acid for several animal species (12-18). However, indolepropionic acid, indolebutyric acid, and indoleacetic acid are inactive (4, 19), the tryptophan metabolites kynurenic acid and xanthurenic acid

are also inactive (2), and kynurenine is either inactive or only slightly active (2).¹ It appears likely, therefore, that none of these compounds is the primary intermediate in the rat in the pathway whereby tryptophan is converted to nicotinic acid derivatives. The present data do not permit an evaluation of the comparative utilization of L- and D-tryptophan by rats deficient or partially deficient in the various vitamins studied. The relatively smaller increases noted when tryptophan is fed as casein rather than as the free amino acid can apparently be explained by the effect of other amino acids contributed by the casein in reducing the amount of nicotinic acid produced (5, 20).

DISCUSSION

The data presented show that several changes in the dietary treatment, particularly with reference to the intake of certain B vitamins and calories, markedly influence the urinary excretion of nicotinic acid metabolites when tryptophan is ingested. It is concluded from the evidence available at the present time that at least a part of this conversion occurs within the tissues of the animal, but that the magnitude of the response is perhaps influenced by the intestinal microorganisms. Microorganisms that normally occur in the intestine have been shown to produce nicotinamide in amounts that vary with the amino acids present in the medium (21, 22). When tryptophan was incubated with mixed bacterial cultures obtained from rat cecum, an increase in the formation of nicotinamide was observed. Certain *coli* strains produce nicotinamide from ornithine, glutamine, and arginine, while other amino acids, including tryptophan, either had no effect or inhibited nicotinamide synthesis.

The pathways involved in the transformation of tryptophan to nicotinic acid by *Neurospora* have been studied in some detail (23-25).

The influence of the intestinal flora on the conversion of tryptophan to nicotinic acid does not appear to be the only factor involved, however. This observation is based on the following information: (a) tryptophan increases the production of nicotinic acid in the developing chick embryo (8); (b) a rapid and large increase in the formation of nicotinic acid occurs when tryptophan is fed to a wide variety of animals with a wide variation in experimental conditions and dietary treatment (1-4, 12-19), and a rapid and large decrease in the urinary excretion occurs when tryptophan is withdrawn from the diet, which is followed by rapid increases when the amino acid is again supplied; (c) certain B vitamin deficiencies, but not all, result in an ineffective conversion of tryptophan to nicotinic acid; (d) intraperitoneal injection of tryptophan results in a considerable increase in the excretion of nicotinic acid metabolites, although smaller than when tryptophan is given orally; and (e) succinylsulfathiazole in the presence of

dietary pteroylglutamic acid does not reduce the amount of nicotinic acid excreted.

The metabolic mechanisms involved in this transformation that are influenced by a deficiency of thiamine, riboflavin, vitamin B₆, and pteroylglutamic acid have not been defined. In view of the large number of chemical changes that are apparently necessary for tryptophan to be converted to nicotinic acid, it may not be too surprising that these vitamins do function in enzyme systems at various stages in the conversion.

It has been shown that a low protein intake reduces considerably the riboflavin and protein in the tissues and, as a consequence, the activity of tissue enzymes (26-33). It is quite likely, therefore, that restriction of the caloric intake, and thereby the protein intake, resulted in a reduction in the effective enzyme concentrations in the tissues and was reflected by the reduction in the excretion of N¹-methylnicotinamide and nicotinic acid when tryptophan was fed. Alternatively, the tryptophan supplements may have been utilized as a source of calories when the food intake was restricted.

SUMMARY

The amount of nicotinic acid derivatives excreted by rats fed tryptophan in addition to purified diets deficient in thiamine, riboflavin, pantothenic acid, pteroylglutamic acid, or vitamin B₆ has been determined.

The urinary excretion of nicotinic acid metabolites when tryptophan was fed was relatively insensitive to the inclusion of succinylsulfathiazole in diets containing pteroylglutamic acid, to the omission of pteroylglutamic acid from the diet, or to the omission of pantothenic acid. Conversely, a marked reduction in the urinary excretion of nicotinic acid occurred when succinylsulfathiazole was added to diets not containing pteroylglutamic acid, and when vitamin B₆, thiamine, or riboflavin was omitted from the diet or when the caloric intake was severely restricted. The implications of these results are discussed.

D-Tryptophan can be effectively utilized by rats fed adequate diets and a summary is included of the results obtained on the urinary excretion of nicotinic acid metabolites when compounds related to tryptophan were tested.

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STUDIES ON MALARIAL PARASITES

IX. CHEMICAL AND METABOLIC CHANGES DURING GROWTH AND MULTIPLICATION IN VIVO AND IN VITRO*

By ERIC G. BALL, RALPH W. MCKEE, CHRISTIAN B. ANFINSEN,
WALTER O. CRUZ, AND QUENTIN M. GEIMAN

(From the Department of Biological Chemistry and the Department of Comparative Pathology and Tropical Medicine, Harvard Medical School, Boston)

(Received for publication, May 6, 1948)

The growth and multiplication of malarial parasites *in vitro* have been described in previous papers of this series (1, 7). Cytological examination was mainly employed as a criterion of growth and multiplication in those studies. When attempts were made to assay the essential nature of the various components of the medium employed, it became apparent that a study of chemical and metabolic changes in addition to cytological observations might permit a better evaluation of the rôle of certain individual nutrients in the growth process of the parasite. We therefore undertook this study of the chemical and metabolic changes occurring during the growth and multiplication of the malarial parasite. In order to establish the normal pattern, values for parasites undergoing growth and multiplication within the host are first presented. Values are then given for parasites grown *in vitro*. The results to be described indicate that parasites grown *in vitro*, even on our complete medium, differ in some aspects of their metabolism from those grown within the animal body.

Methods

The general procedures for handling and inoculation of monkeys with *Plasmodium knowlesi*, the drawing of blood samples, the counting of blood cells and parasites, and their evaluation have been described in Paper VII of this series (7).

Methods for the determination of glucose and lactate and measurement of oxygen consumption were the same as those described in Paper VI of this series (13). Lactate consumption is calculated by assuming that each glucose molecule that disappears yields 2 molecules of lactate. From this figure, the amount of lactate that actually accumulates is then subtracted.

Inorganic, acid-soluble, 15 minute-hydrolyzable, and total phosphorus analyses were made by the Fiske and Subbarow method (6) with semimicro

* The work described in this paper was done under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the President and Fellows of Harvard College during the years 1944-45.

quantities of materials. The difference between total P and the sum of acid-soluble P and phospholipide P is expressed in our tables as nucleic acid phosphorus. We recognize that this fraction may contain some phosphoprotein. We believe, however, in the case of the parasitized cell, that no great error arises in treating it as all nucleic acid phosphorus in our discussions.

Flavin-adenine dinucleotide was determined by means of its ability to restore oxygen consumption to a coenzyme-free D-amino acid oxidase system in the manner first described by Warburg and Christian (22). A sample of flavin-adenine dinucleotide kindly supplied by Professor Warburg was used as a standard, the purity being checked by us spectrophotometrically, using the β value (*cf.* (2)) at λ 450 $m\mu$ as equal to 2.60×10^7 . The cells from 4 cc. of whole blood were used for analysis. The cells were centrifuged off, the serum discarded, and the cells laked with 6 cc. of water. Since the serum contained very little flavin-adenine dinucleotide, washing of the cells before laking was omitted. The mixture of laked cells was then placed in a water bath held at 90° and stirred intermittently for 10 minutes. The mixture was then promptly cooled to room temperature and centrifuged, and the supernatant decanted. The residue was washed with 3 cc. of water and centrifuged, and the supernatant pooled with the first extract. This process was repeated twice more, and the combined supernatants evaporated to dryness in a vacuum desiccator in the cold room. The dry residue so obtained was extracted with water just before analysis, and made up to a total volume of 2.0 cc. An aliquot of 0.5 cc. usually sufficed for a determination.

The analytical methods employed in the assay of the lipid fractions were modifications of existing techniques. To permit duplicate estimations of total fatty acids and phospholipide phosphorus, it was found essential to increase the sensitivity of the techniques. The methods to be described are designed for 1.5 to 3.0 cc. samples of blood. Best results are obtained when the degree of parasitization is greater than 5 per cent, since at lower levels the differences between samples of parasitized cells and normal cells do not permit an accurate evaluation of the portion contributed by the parasites themselves.

Lipide extraction is performed by centrifuging the blood samples (1.5 to 3.0 cc.) and discarding the plasma. Care must be taken not to disturb the surface of the packed cells, since it is in this stratum that the lighter, lipid-rich cells predominate. The cells are washed with 4 volumes of ice-cold 0.85 per cent saline, recentrifuged, and the supernatant again discarded. The cells are then transferred to special extraction vessels, prepared from narrow necked, round bottomed centrifuge tubes calibrated at the neck to contain 50 cc. The transfer is made with a Pasteur pipette, and the cells

adhering to the pipette and centrifuge tube are washed into the extraction vessel with a minimum of distilled water (two 0.1 cc. portions are adequate). About 30 cc. of 3:1 alcohol-ether mixture are then added rapidly, and the vessel stoppered and shaken vigorously. This procedure generally yields a fine, easily extracted suspension. If large, gummy clots form, they must be thoroughly broken up with a ball-ended stirring rod. The vessel is filled to the mark with alcohol-ether mixture and shaken at intervals for about an hour. Control experiments indicate that the extraction is complete after this time interval. Warming the tube is not required. The extract can now be filtered through fat-free filter paper into 125 cc. Erlenmeyer flasks with the usual precautions against evaporation. As an alternative, the vessels may be centrifuged sharply and aliquots removed directly. The volume contributed by the solids of the red cell is less than 1 per cent of the total volume and is disregarded in the calculations.

In the method for the determination of phospholipide phosphorus the reagents of Fiske and Subbarow (6) are employed. Duplicate 3 cc. aliquots of the total lipid extract are pipetted into 10 cc. calibrated test-tubes. These are placed in an ammonium sulfate-sulfuric acid bath (12) and the solvents are evaporated off. Then 0.4 cc. of 10 N sulfuric acid is added and the temperature of the bath slowly raised to about 120°, at which point a few small drops of fuming nitric acid are added down the side of the tube in such a manner as to wash the walls. The temperature is allowed to rise to 190°, but not above 200°. If charring still persists, another drop or two of concentrated nitric acid are added. The tube must remain in the bath at this temperature until no further oxides of nitrogen can be detected. The tube is cooled, its contents diluted to about 8 cc., swirled to mix, and 0.3 cc. of ammonium molybdate solution added. After mixing again, 0.4 cc. of the aminonaphtholsulfonic acid reducing mixture is added. The contents of the tube are mixed at once, diluted to 10 cc., remixed, and, after standing for a minimum of 15 minutes, read in a Klett-Summerson photoelectric colorimeter. A reagent blank containing all the above components with the exception of phosphate is run as a base-line for the calculations. The standard tube for comparison contains 0.0200 mg. of inorganic phosphorus.

For the determination of total fatty acids, duplicate 15 cc. aliquots of the total lipid extract are pipetted into 50 cc. Erlenmeyer flasks. The solvents are evaporated off on a steam bath. 2 cc. of 1 N alcoholic KOH are added, and the lipides saponified by heating on the steam bath for 45 minutes. If more than half the alcohol is lost during this process, the volume is brought back to about 2 cc. After saponification the solution is transferred quantitatively to large test-tubes (about 25 × 200 mm.) and the solvent evaporated in a boiling water bath. While the contents of the

tube are still warm, 2.5 cc. of 1 N sulfuric acid are added. After shaking, 10 cc. of petroleum ether are added to extract the fatty acids. The tubes are placed in the refrigerator and allowed to stand overnight. During the storage period, sodium sulfate may precipitate. Extraction periods up to 3 days were shown not to yield any higher results than those obtained by this procedure.

The petroleum ether extract is now washed thoroughly by rinsing down the sides of the tube six times with distilled water. The aqueous phase is removed as completely as possible after each rinse by means of a long capillary tube of about 1.5 mm. outside diameter, attached to an aspirator and reaching to the bottom of the tube. After the final rinse, the tube is removed and rinsed off with petroleum ether. The capillary tube should be full of water during removal to prevent loss of the petroleum ether phase by capillarity. The petroleum ether is evaporated off and the residuum is dissolved in 5.0 cc. of 95 per cent ethyl alcohol redistilled from NaOH pellets. A uniform sized drop of alcoholic phenolphthalein is added as indicator along with 0.5 cc. of distilled water. High and variable blank titrations occur in the absence of the addition of this quantity of water. The alcoholic solution of fatty acids is then titrated with 0.05 N NaOH, by use of a 1 ml. burette with a long capillary tip which reaches below the surface of the alcoholic solution. In analyses for the fatty acids in the red cells from 3.0 cc. of blood with a normal hematocrit, the total titration varies between about 0.09 and 0.20 cc., depending on the degree of parasitization.

Hemoglobin and hematin contents of normal and parasitized cells were determined by a spectroscopic method with the Beckman quartz spectrophotometer. Hemoglobin was determined on samples of cells laked in distilled water, while total hematin was determined by laking with alkaline alcohol (0.04 gm. of potassium hydroxide in 80 cc. of ethyl alcohol). The exact procedure of measurement varied with the type of experiment to be performed and will be described in more detail in presenting the results.

Results

Chemical and Metabolic Changes Occurring during Growth and Multiplication of Plasmodium knowlesi in Vivo

It is necessary to express our results in terms of a unit amount of red cells throughout, since it is with this component of whole blood that our results deal and a wide fluctuation of plasma to red cell volume is encountered from one blood sample to another. We have chosen as our unit 5×10^{12} total red blood cells, parasitized and normal. This is the number of cells present on the average in 1 liter of normal whole blood. This unit

permits the results to be expressed in mm or mg. as whole numbers. The expression of results in terms of a number of red cells rather than per volume of red cells facilitates the calculation of results per parasitized cell, since the parasite count is expressed in percentages. Hematocrit readings are, however, also given so that if the need arises calculations on this basis may be made by the reader from the data. The hematocrits and red cell counts, however, show agreement in the extent of their changes usually to within ± 5 per cent. The general conclusions to be drawn will not be markedly affected, therefore, by the method of expression of the results.

Results have also been expressed in the *in vivo* experiments in terms of 5×10^{12} parasitized cells. These figures have been calculated according to the following formula.

mm/ 5×10^{12} parasitized cells

$$= \frac{\text{mm}/5 \times 10^{12} \text{ total cells} - (\text{mm}/5 \times 10^{12} \text{ normal cells} \times \% \text{ unparasitized cells})}{\% \text{ parasitized cells}}$$

In this calculation, we have assumed that, in a sample of parasitized blood, the unparasitized cells have the same chemical properties as those in a normal blood sample. We have no proof for this assumption.

In Tables I and II, the results of experiments are given which were designed to follow changes in the various components and metabolic rates under study as the number of parasites increased. In these experiments, a sample of blood was withdrawn from a monkey just before inoculation with *Plasmodium knowlesi*. Samples were again drawn at appropriate times after the parasitemia was well established as shown by hematological evaluation of samples of ear blood. Attempts were made to select infected animals in which the asexual stages of the parasites were "in step," and to draw samples at approximately the same point in the daily cycle so that parasite size would remain nearly constant throughout.

In Table III, the results of experiments are given which were designed to follow the chemical changes that occur as the parasite grew from young forms to old. Considerable changes also occurred in parasite numbers during the interval between the collection of the two blood samples. In this experiment, therefore, the values calculated for parasitized cells are a truer index of the changes occurring as a result of parasite age alone. In making these calculations, we have used the average values for normal monkey blood given in the foot-note to Table III, since no normal blood samples were withdrawn from the animals used in these experiments.

The results presented in Tables I to III may be best considered together. The most striking changes have occurred in the rates of utilization of oxygen, glucose, and lactate. The data in Tables I and II show that a 25-

noted that in all cases the ratio of the rate of utilization of oxygen to glucose on a millimolar basis is approximately unity. Since the complete combustion of 1 mm of glucose to CO_2 and H_2O requires 6 mm of oxygen, it is obvious that complete combustion in these experiments can account for an amount of glucose equivalent to only one-sixth of the total that disappears.

The figures for lactate accumulation indicate that a large part of the glucose utilized can be accounted for by this glycolytic product. It may be calculated from the data given that 54 to 82 per cent of the glucose that disappears in heavily parasitized blood appears as lactate. If we add to

TABLE II

Chemical and Metabolic Changes Occurring in Red Blood Cells As Number of Parasites Increases in Vivo

Monkey 78; 6.3 kilos; infected with *Plasmodium knowlesi*.

	Feb. 8, 1945		Feb. 13, 1945		10.55 a.m., Feb. 14, 1945		11.00 a.m., Feb. 15, 1945	
Blood sample, cc.	12		14				109 *	
Red cells, per c.mm.	6.08×10^6		5.23×10^6		3.80×10^6		1.56×10^6	
Hematocrit, %	42.6		35.6		28.4		12.0	
Parasites, %	0		4.2		16.8		50.0	
Rings			0.0		1.0		2.0	
Trophozoites, early			60.0		39.5		44.0	
" late			27.0		48.5		43.5	
Schizonts			9		10.0		9.5	
Segmenters.			1		0.5		1.0	
	Total	Para- sitized	Total	Para- sitized	Total	Para- sitized	Total	Para- sitized
mm per hr. per 5×10^{12} cells								
O ₂ consumption	0.87		1.07	5.72	5.20	26.7	21.82	42.8
Glucose consumption	0.51		1.28	18.8	7.90	44.5	18.14	35.8
Lactate "	0.0		0.0	0.0	2.78	16.6	9.97	19.9
" accumulation	1.02		2.56	37.6	13.02	72.4	26.31	51.7
mm per 5×10^{12} cells								
Fatty acids	3.9		4.6	20.7			11.1	17.5
Total P	7.5		9.6	57.2	10.7	26.8	24.4	41.3
Acid-soluble P	4.77		5.59	25.5	5.86	11.4	9.45	14.1
15 min.-hydrolyzable P	0.89		1.01	3.81	1.18	2.62	2.43	3.98
Phospholipide P	1.80		1.7		2.4	4.8	4.5	7.1
Nucleic acid "	0.90		2.3	34.3	2.4	9.8	10.4	19.9
Mg. per 5×10^{12} cells								
Flavin-adenine dinu- cleotide	0.14		0.27	3.33	0.40	1.67	1.12	2.10

* Terminal.

this the 17 per cent reacting with oxygen to form CO₂, we can account for 71 to 89 per cent of the glucose utilized by these various bloods. The pattern of carbohydrate metabolism in *Plasmodium knowlesi* thus resembles that found for *Plasmodium gallinaceum* by Silverman *et al.* (18).

TABLE III
*Chemical and Metabolic Changes Occurring in Red Blood Cells As Parasite Size
 Increases in Vivo*

	Monkey 86; 6.1 kilos				Monkey 98; 4.4 kilos			
	4 20 p.m., Apr. 11, 1945		1.00 p.m., Apr. 12, 1945		10.00 a.m., June 12, 1945		9.00 p.m., June 12, 1945	
Blood sample, cc...			110*		17		17	
Red cells, per c.mm...	3.45 × 10 ⁶		2.08 × 10 ⁶		3.81 × 10 ⁶		2.37 × 10 ⁶	
White cells, per c.mm.	13,200		14,400		34,400		38,700	
Hematocrit, %	28.0		15.5		32.3		21.3	
Parasites, %...	33.7		47.8		47.4		34.4	
Rings	41		6		4		2	
Trophozoites, early	52		27		88		16	
" late	3		40		1		71	
Schizonts	2		25		2		4	
Segmenters	2		2		1		0.5	
	Total	Para- sitized†	Total	Para- sitized†	Total	Para- sitized†	Total	Para- sitized†
mm per hr. per 5 × 10 ¹² cells								
O ₂ consumption	6.48	17.9	16.70	34.2	8.78	17.8	17.95	51.0
Glucose consumption	6.44	17.9	14.14	28.9	10.77	22.1	18.27	52.0
Lactate	4.83	14.3	13.07	27.3	5.83	12.3	9.37	27.2
" accumulation	8.05	21.5	15.21	30.5	15.71	31.9	27.71	76.8
mm per 5 × 10 ¹² cells								
Fatty acids					11.8	19.7	10.0	20.1
Total P	12.77	19.0	22.33	36.3	15.43	21.9	17.45	32.4
Acid-soluble P	7.18	8.35	8.48	10.5	7.10	7.67	7.61	9.57
15 min.-hydrolyzable P	1.78	2.7	2.46	3.7	1.68	2.09	2.28	4.1
Phospholipide P	3.1	5.4	4.4	7.1	3.7	5.7	4.1	8.3
Nucleic acid	2.5	5.3	9.5	18.7	4.6	8.5	5.8	14.5
Mg. per 5 × 10 ¹² cells								
Flavin-adenine dinucleotide	0.54	1.28	0.84	1.57	0.53	0.93	0.85	2.15

* Terminal.

† The values for parasitized cells were calculated according to the method given in the text, with the following values (mm) for 5 × 10¹² normal red cells: O₂ consumption 0.70; glucose consumption 0.60; lactate consumption 1.20 mm per hour; fatty acids 4.7, total P 9.61, acid-soluble P 6.60, 15 minute-hydrolyzable P 1.31, phospholipide P 1.91 mm; flavin-adenine dinucleotide 0.17 mg.

On the basis of the values calculated for 100 per cent parasitized cells, it becomes obvious that each parasitized cell may use from 25 to 75 times the oxygen and glucose required by a normal red blood cell. The parasitized cell also, as noted before (13, 23), utilizes lactate, which the normal cell

does not appear to do to any great extent. Even so, it is obvious from the data presented here that the metabolism of glucose by the parasitized cell is predominantly of the anaerobic type, such as is found in the normal red cell. Indeed, as shown earlier (1), it is possible for *Plasmodium knowlesi* to grow and multiply *in vitro* under practically anaerobic conditions.

In the various chemical components that have been studied, there is a definite increase in all of them, with the possible exception of acid-soluble phosphate, as the parasite number or size increases. The total phosphorus value of the cells from highly parasitized samples is double or triple that of normal cells. As might be expected, the greatest part of this increase is to be found in what we have chosen to call the nucleic acid phosphorus fraction (see "Methods"). In the normal cell, this fraction constitutes, on the average, only about 11 per cent of the total P. In the parasitized cell, it comprises from 28 per cent of the total, when the parasites are very young, to as high as 64 per cent, when the parasites are fairly well developed. Part of the increase in total P is due to an increase in the 15 minute-hydrolyzable fraction. If, as is customary, we assume that the chief component in this P fraction is adenosine triphosphate, then the data show that the parasitized cell contains between 2 and 4 times the amount of this high energy phosphate compound as does the normal red cell. In view of the fact that glucose utilization by the parasitized cell is 25 to 70 times that of the normal cell, either there must be a more rapid turnover of adenosine triphosphate in parasitized cells, or some other energy transfer mechanism is present.

An increase in the phospholipide P also contributes a share to the increase in total P. It may be calculated from the data in Tables I to III that the parasitized cell contains 2.8 to 4.4 times the phospholipide P found in the normal red cell, the range representing the difference between young and old forms. Concomitant with the increase in phospholipide P, there is an increase in fatty acid content. The increase in fatty acid content parallels the phospholipide P changes, so that the ratio of mm of fatty acid to mm of phospholipide P tends to remain within the range of 2.5 to 3.0. This aspect will be dealt with again in a later section.

The changes in the acid-soluble P fraction of parasitized cells are not uniform in the various experiments. The data in Table I show little change in this P fraction. Small increases over values for normal red cells may be observed in the data given in Tables II and III. The acid-soluble P fraction includes only organic phosphate. Values for the 15 minute-hydrolyzable fraction, as mentioned above, rise with parasitization of the red cell. The inorganic phosphate content of the red cell tends to decrease upon parasitization (13). In many normal cells, glycerol phosphates comprise a large portion of this P fraction, as shown by Rapoport and Guest

(15). It seems likely that changes in this component may be responsible for the variation in results observed here, but we have no data bearing upon this point.

The remaining component that was studied is flavin-adenine dinucleotide. The concentration of this respiratory coenzyme also rises as the parasite size or number increases. From the values calculated in the various tables, the parasitized cell is seen to contain from 6 to 15 times the amount of this coenzyme that is found in a normal red cell. As stated above, the respiration rate of the parasitized cell is 25 to 75 times that of the normal cell. There is thus not a direct relation between the increases in this coenzyme and respiratory rate. The fact that the content of flavin-adenine dinucleotide is increased in the parasitized cell is suggestive that it plays a rôle in the parasites' economy. This raises the question of the relation of the action of atabrine to the functioning of this coenzyme. Attempts (8, 10) have been made to correlate the action of atabrine with an inhibition of enzyme systems whose coenzyme is flavin-adenine dinucleotide. The concentration of atabrine needed, however, to inhibit such enzyme systems is somewhat higher than is needed to suppress parasite growth *in vivo*. In view of the findings reported here, it seems possible that atabrine may function by blocking the synthesis of this coenzyme rather than by competing with it for the enzyme molecule.

Chemical and Metabolic Changes Occurring during Growth and Multiplication of Plasmodium knowlesi in Vitro

Oxygen Consumption—One of the most striking changes that occurs in red blood cells as they become parasitized is the increase in oxygen consumption. As pointed out above, for parasites grown *in vivo* this change is readily noticeable whether the size of the parasite or the number of parasites per unit number of cells increases. For parasites grown *in vitro*, we have consistently failed to detect such a concomitant increase in oxygen consumption with increase in parasite size or number. The results of representative experiments are presented in Table IV. Oxygen consumption is expressed in mm per hour per 5×10^{12} cells so as to be directly comparable with the *in vivo* experiments and to cancel out the fluctuations in total red cell count that occur in the rocker perfusion type of culture apparatus used in these experiments. The culture medium employed in Experiments R.P.A.-9-3, 43, and 57-1 is that given in Paper I of this series (3) and contained proteose peptone. In Experiment R.P.A.-57-4, both proteose peptone and *p*-aminobenzoic acid were employed, while in Experiment R.P.A.-58, only the latter was included, at a concentration of 10 γ per cent. Otherwise, the composition of the medium was the same in all experiments. From the *in vivo* experiments reported above, the increase in oxygen con-

sumption should be about the same as the increase in parasite number. Thus, in Experiment R.P.A.-9-3, a 3-fold increase in oxygen consumption might be expected to occur at the end of the experiment, when the parasite percentage had increased from 13.2 to 44.3. The oxygen consumption has, however, changed but little throughout the experiment. The only *in vitro* experiments in which an increase of oxygen consumption was observed were Experiments R.P.A.-57-1 and 57-4 reported in Table IV. It is to be noticed that the multiplication of parasites in both of these experiments was less than 2-fold and they are therefore decidedly below par from this standpoint. In Experiment R.P.A.-57-4 the inclusion of both proteose peptone

TABLE IV
Respiration of Parasites (Plasmodium knowlesi) Grown in Vitro

Experiment	Time	O ₂ uptake, mm per hr per 5 × 10 ¹² cells	Blood count						
			Red blood cells	Per cent parasites					
				Total	Rings	Troph- ozoites	Schiz- onts	Seg- menters	Degen- erate
	hrs.		millions per c.mm						
R.P.A.-9-3	0	5.5	3.96	13.2	13.5	72.5	13.0	0.5	
	4	5.3	3.80	13.8	12.0	45.0	36.0	2.0	
	6	5.7	3.50	22.0	23.0	24.5	35.0	4.0	
	10	4.7	3.89	27.0	29.0	24.0	30.0	7.0	
	14	4.6	4.13	30.6	43.5	41.0	2.5		
	20.5	5.2	3.33	44.3	4.5	76.0	2.0		
R.P.A.-43	0	1.35	4.78	2.6	0	64.0	35.0		
	22	1.58	4.27	10.1	8	89.0	2.0		
R.P.A.-57-1	0	1.19	5.57	7.4	30.0	56.0	4.0		
	23	2.09	4.07	11.8	5.0	54.0	22.0		18.0
R.P.A.-57-4	0	1.19	5.57	7.4	30.0	56.0	4.0		
	23	3.13	4.16	13.0	3.0	58.0	28.0	5.0	2.0
R.P.A.-58	0	1.87	4.33	3.6	3.0	86.0	7.0	2.0	
	23.5	1.72	4.03	13.2	2.0	94.0			3.0

and *p*-aminobenzoic acid in the medium may be a contributing factor to these findings.

We have no explanation for this failure of parasites grown *in vitro* to increase their oxygen requirements other than to postulate that our culture conditions are not satisfactory for aerobic growth of the parasites. We have shown previously (1) that parasites can grow and multiply *in vitro* under nearly anaerobic conditions. It is possible that in our culture experiments in which the glucose concentration is maintained at high levels (250 mg. per cent) the parasites elect to obtain most of their energy by anaerobic processes. On the other hand, a contributing cause may be a

lack of certain heavy metals or other components in our medium that are needed for the formation of the respiratory enzymes of the parasite cell. Certainly the lack of hemin seems an unlikely cause (see below).

Glucose and Lactate Metabolism and Phosphorus Fractions—In Table V are presented data on the changes in glucose and lactate metabolism and on phosphorus fractions which occur in normal and parasitized blood cultured for 21 to 24 hours *in vitro* by the rocker perfusion technique described in Paper VII of this series (7). A comparison with the data obtained on parasites grown *in vivo* indicates that a somewhat different pattern exists in the *in vitro* experiments in the case of the phosphorus fractions. There occurs uniformly a drop in the total acid-soluble phosphorus of the red cells of parasitized blood during the cultivation period. The magnitude of this fall varies from experiment to experiment and may even occur during incubation by the same technique of normal blood, as indicated by the data for Experiment R.P.A.-60-3 given in Table V. This decrease in total acid-soluble phosphorus is reflected in the values for total organic P which show less change than those observed in the case of parasites grown *in vivo*. However, in three of the four experiments for which data are presented in Table V some increase in total organic P occurred in spite of the drop in the acid-soluble P fraction. There is thus a definite increase in acid-insoluble organic P (phospholipide + nucleic acid P) during growth and multiplication of the parasites *in vitro*. As in the case of the parasites grown *in vivo*, the 15 minute-hydrolyzable P fraction also increases as the parasite number increases. The chief difference between the parasites grown *in vivo* and *in vitro* thus lies mainly in the acid-soluble fraction. This suggests that parasites grown *in vitro* may make more use of the acid-insoluble phosphorus fraction of the red cell to synthesize their needed phosphorus compounds than do parasites grown *in vivo*.

The data for glucose and lactate utilization indicate that the growth and multiplication of parasites *in vitro* are also accompanied by an increase in the metabolic rates of these two compounds. The data on glucose and lactate utilization presented in Table V were obtained by two different techniques. In Experiments R.P.A.-41, 60-3, 40-1, 60-2, the data were obtained by an analysis of the culture medium before and after its passage through a cellophane tubing immersed in the parasitized blood sample.

In the rocker perfusion technique, which has been fully described elsewhere (7), about 1 cc. per minute of nutrient fluid flows through approximately 15 cc. of blood. The effluent fluid in these experiments was collected under paraffin oil and toluene. At the times stated in Table V, the contents of the collecting bottle were removed and well mixed, the volume determined, and glucose and lactate determinations made. At the same time, a blood sample was also taken for glucose and lactate analysis. The glucose con-

tent of the inflowing medium was also determined. Since the volume of blood and its red blood cell count were also known, the average hourly utilization figures given in Table V were calculated for each of the time intervals represented there. The utilization values obtained by this technique reach much higher levels than those given by the tonometer technique. This seems to be especially true for values obtained for the

TABLE V

Changes in Glucose and Lactate Utilization and Phosphorous Fractions during Growth of Plasmodium knowlesi in Vitro

Experiment	Time	Parasites	Phosphorus				Utilization	
			Total organic	Acid-soluble organic	15 min.-hydrolyzed	Acid-insoluble organic	Glucose	Lactate
	hrs.	per cent	mm per 5×10^{12} cells				mm per hr. per 5×10^{12} cells	
R.P.A.-41	0	0	12.9	7.43		5.5	0.51	0
	24	0	14.7	8.33		6.4	0.62	0
R.P.A.-60-3	0	0	11.1	6.54	1.34	4.6	0.46	0
	21	0	9.8	4.97	1.08	4.8	0.69	0
R.P.A.-40-1	0	5.0	13.5	6.56		6.9	2.40	1.10
	23	22.6	14.3	4.49		9.8	5.15	2.01
R.P.A.-60-2	0	4.4	11.1	6.07	1.19	5.0	2.26	3.59
	21	7.0	12.2	4.27	1.22	7.9	2.41	3.15
R.P.A.-32-2	0	6.0		6.03	0.92		*	*
	4.5	6.8					3.11	3.40
	8.5	17.2					3.46	3.40
	22	20.4		3.92	1.48		13.90	18.40
R.P.A.-36-1	0	3.0	13.2	8.27	1.43	4.9	*	*
	4.5	9.6					2.95	3.84
	10	16.1					4.03	4.94
	23	12.6	11.1	5.50	1.71	5.6	4.27	0.96
R.P.A.-38-1	0	4.3	10.9	6.44	1.07	4.5	*	*
	4	5.0					8.8	13.1
	10	12.0					14.2	24.3
	22	23.0	14.2	6.10	2.21	8.1	20.4	26.7

* Values calculated from analysis of the culture medium before and after its perfusion through the culture (see the text).

periods just after segmentation and reinvasion have occurred. We have no explanation for this pronounced difference in results. As far as we could ascertain, there was no bacterial contamination of the collected perfusion fluid. The chief difference in the two techniques lies in the fact that in one, the analysis of the perfusion fluid, the metabolism of the parasites *during* segmentation and reinvasion is measured. In the other, metabolism is

measured only during a short period in which the parasite population is static. Whether this fact can account for the large differences observed must await further experimentation. The observations of Velick (21) are of interest in this connection. He showed that the oxygen consumption of *Plasmodium cathemerium* is greatly accelerated when nuclear division begins and concomitantly the respiratory quotient increases.

One point is, however, clear. The multiplication of parasites *in vitro* is attended by an increase in the rate of glucose utilization. Indeed, with the rocker dilution technique for cultivation, analysis of glucose in the culture has been a routine procedure with us, and the extent of multiplication in such experiments can be fairly accurately predicted from the magnitude of glucose utilization throughout the culture period.

Phospholipide Phosphorus and Fatty Acid Relationships in Parasitized Red Cells Grown in Vivo and in Vitro—The importance of the phosphatides in relation to fat metabolism in the mammalian organism suggested a study of the relationship of phospholipide phosphorus and fatty acid content of parasitized cells. In Table VI are given data on these two components in normal red blood cells of the monkey and in cells with varying percentages of parasites as drawn directly from monkeys infected with *Plasmodium knowlesi*. The molar ratio of fatty acids to phospholipide P in normal cells is on the average 2.46. In liver, this ratio averages 7.4 according to calculations we have made on the data of Handler (9) and Stetten and Grail (20). If all the fatty acids were incorporated into phosphatides of the lecithin or cephalin type, this ratio would be 2.00. On this basis, it is apparent that free fatty acid or neutral fat constitutes only a small portion of the total fatty acids of the normal monkey red blood cell. The same may be said to be true for parasitized cells. It is of interest in this connection that *Plasmodium knowlesi* is able to oxidize glycerol at a very rapid rate (13, 23). Whether this ability to destroy glycerol is related to the low neutral fat content of the organism is a matter for further exploration. From the data given in Table VI, it is clear that an increase in both phospholipide P and fatty acid content occurs as the per cent of parasitized cells in the blood sample rises. However, there is little, if any, change in the ratio of these two components. The data indicate on the average an increase in the ratio of 7 per cent, which is probably within the experimental error.

Several experiments have also been made on parasitized cells grown *in vitro*. The results are given in Table VII. There is an indication from the two experiments given here that an abnormal situation may exist in the relationship of fatty acids to phospholipide P in parasitized cells grown *in vitro*. In both experiments, an increase in the ratio of fatty acids to phospholipide P occurred during the 23 hour culture period. It is to be noted that in each case, though the phospholipide P content increased, the

fatty acid content increased even more. Indeed, the value for fatty acids found at the end of Experiment R.P.A.-36-1 is the largest encountered and exceeds the values reported in Table VI for blood samples containing 4 times as many parasites. More data are admittedly needed to establish this point. There is, however, a suggestion here that our present culture medium may need modification with regard to the choline, methionine, or

TABLE VI
Phospholipide Phosphorus and Fatty Acid Content of Normal and Parasitized (Plasmodium knowlesi) Monkey Red Blood Cells

	Blood sample No.	Parasites	Phospholipide P	Fatty acids	Ratio, $\frac{(4)}{(3)}$
	(1)	(2)	(3)	(4)	
		<i>per cent</i>	$\frac{\text{mm per}}{5 \times 10^{12} \text{ cells}}$	$\frac{\text{mm per}}{5 \times 10^{12} \text{ cells}}$	
Normal	1		1.80	3.9	2.17
"	2		2.08	5.0	2.40
"	3		1.78	4.9	2.75
"	4		1.89	4.2	2.22
"	5		1.95	4.8	2.46
"	6		1.83	4.9	2.70
"	7		2.00	5.2	2.60
Average			1.91	4.7	2.46
Parasitized	1	7.4	2.59		
"	2	16.8	2.40		
"	3	17.3	2.70	7.3	2.70
"	4	19.0	2.59		
"	5	27.0	2.67		
"	6	28.0	2.80	7.2	2.57
"	7	33.7	3.1		
"	8	34.4	4.1	10.0	2.44
"	9	40.6	4.0	9.4	2.35
"	10	45.0	4.2	11.8	2.81
"	11	47.4	3.7	11.8	3.16
"	12	50.0	4.5	11.1	2.47
Average					2.64

other components involved in fat metabolism, or that the high glucose content of the medium (250 mg. per cent) may be causing abnormal fatty acid synthesis.

Flavin-adenine Dinucleotide Changes in Normal and Parasitized Blood in Vitro—The definite increase observed in the flavin-adenine dinucleotide content of parasitized blood as the parasites either increase in size or number *in vivo* indicates that this coenzyme is essential to the growth of the

TABLE VII

Phospholipide Phosphorus and Fatty Acid Changes in Parasitized Cells (Plasmodium knowlesi) Grown in Vitro

Experiment (1)	Time (2)	Parasites (3)	Phospholipide P (4)	Fatty acids (5)	Ratio, $\frac{(5)}{(4)}$
	hrs.	per cent	mm per 5×10^{12} cells	mm per 5×10^{12} cells	
R.P.A.-36-1	0	3.0	2.35	5.2	2.21
	23	12.6	2.81	12.2	4.34
R.P.A.-39	0	6.6	2.08	5.2	2.50
	23	11.6	2.38	6.8	2.86
R.P.A.-50	0	6.3	2.02		
	22	28.6	2.24		

TABLE VIII

Flavin-adenine Dinucleotide Changes in Normal and Parasitized (Plasmodium knowlesi) Blood Cultivated in Vitro at 38°

Blood	Experiment	Time	Parasite count		Flavin-adenine dinucleotide
		hrs.		per cent	mg. per 5×10^{12} cells
Parasitized monkey	R.P.A.-58	0	Total	3.6	0.37
			Rings	3.0	
			Trophozoites	86	
			Schizonts	7	
			Segmenters	2	
		23.5	Total	13.2	0.48
			Rings	2	
			Trophozoites	94	
			Gametocytes	1	
			Degenerate	3	
Normal monkey	R.P.A.-61	0		0	0.29
		24.0		0	0.38
" human	"	0		0	0.37
		24.0		0	0.54

The rocker perfusion apparatus was employed and a culture medium containing 500 γ of riboflavin, 500 γ of ribose, and 250 γ of adenine per liter. For further details of culture technique, see previous papers (1, 7).

parasite. Similar increases in the amount of this coenzyme can be observed in parasitized blood cultured *in vitro*. The results of an experiment showing such a change are given in Table VIII. The parasite count changed from 3.6 to 13.2 per cent in this experiment and a 30 per cent in-

crease in the coenzyme content resulted. This change is comparable to that observed in the *in vivo* experiment reported in Table II in which a 48 per cent increase in flavin-adenine dinucleotide resulted as the parasite count increased from 4.2 to 16.8 per cent. It should be noted, however, that increases in flavin-adenine dinucleotide content of this magnitude may also be observed in normal blood cultured in the same fashion for 24 hours. The results of an experiment with both normal monkey and human blood in which such a change is observed are also given in Table VIII. The synthesis of the flavin coenzyme by normal human blood cells has been observed previously by Klein and Kohn (11). It is thus not possible to decide from the cultivation experiments *in vitro* whether the malarial parasite possesses the ability to carry on the synthesis of this coenzyme by itself or is dependent upon its host red cell for this important function. The action of atabrine upon the ability of the normal red blood cell to perform this synthesis would be of interest.

Conversion of Hemoglobin to Hematin by Parasites in Vivo and in Vitro—The ability of the malarial parasite to split off hematin from the hemoglobin of the host red cell and to deposit it as an insoluble pigment within its own cell has long been recognized. Sinton and Ghosh (19) have reviewed the early work in this field and, most recently, Rimington *et al.* (16) have confirmed the conclusion of earlier workers that the pigment deposited within the parasite is hematin. Little work has, however, been done on the quantitative aspects of this conversion. We have, therefore, attempted a few experiments along these lines.

In Table IX are presented the results of an experiment performed on a monkey infected with *Plasmodium knowlesi*. Samples of blood were drawn at the beginning of a cycle when the parasites were young and then at two later intervals during the cycle as the parasites developed to maturity. The hemoglobin content of the blood samples was determined by laking a known volume of blood with water, diluting to a constant volume, centrifuging off cellular and parasite debris, and measuring the optical density of the supernatant at the two chief wave-lengths in the visible region of oxyhemoglobin. Alkaline hematin was determined in a similar fashion with alkaline alcohol (0.04 gm. of KOH in 80 per cent ethyl alcohol) to lase the cells. The hemoglobin of the cells is thereby converted to hematin and any preformed hematin in the parasites is also extracted; total hematin combined or free is thus determined.

The values for the optical densities given in Table IX are expressed in terms of a unit number of total red blood cells (parasitized and normal) in order that hemoglobin and hematin concentrations of the different samples may be directly compared. It is clear that per unit number of cells there has been a decrease in hemoglobin concentration but no change in total

hematin concentration as the experiment progressed. Since this decrease in hemoglobin has occurred concomitantly with a small decrease in per cent of parasitized cells, it can be attributed entirely to hemoglobin destruction accompanying growth of the parasite from a pre-ring and early trophozoite form to a schizont or segmenter. The total decrease in hemoglobin concentration is of the order of 17 per cent. The maximum to be expected would be 21 per cent if it is assumed that all the hemoglobin in each parasitized cell is entirely destroyed. Since the observed value is 80 per cent of the maximum theoretical value, it is evident that during its life cycle, the malarial parasite destroys nearly all the hemoglobin in its host cell. By

TABLE IX

Hemoglobin Destruction during Growth in Vivo by Plasmodium knowlesi
Monkey 118.

	2 p.m., Nov. 5, 1945	9 45 a.m., Nov. 6, 1945	3 p.m., Nov. 6, 1945
Red cells, per c.mm. $\times 10^{-6}$	2.96	1.71	1.49
Parasites, %	25.5	21.7	21.2
Pre-rings, %	25		2
Rings, %	2		
Trophozoites, early, %	59	20	12
" late, %	2	52	21
Schizonts, %	7	24	40
Segmenters, %	3		21
Gametocytes, %	1		
Extracellular, etc., %	1	4	4
HbO ₂ ,* λ 540 m μ	0.71	0.61	0.59
" * " 576 "	0.74	0.64	0.61
Hematin,* " 593 "	0.44	0.43	0.44

* The values are expressed as the readings $\log I_0/I$ given in a Beckman spectrophotometer by 10^8 laked red cells per 3 ml. of total volume in a cell with a 1 cm. light path. See the text for further details.

the same token, it may be said that the parasite retains within its cell all of the hematin that it splits off from hemoglobin.

The process of hemoglobin destruction by parasitized red cells may also be observed *in vitro*. In Table X are given the results of an experiment demonstrating this point. The parasitized cells were harvested by the method for differential sedimentation of parasitized cells from normal cells described in Paper VII of this series (7). The cell concentrate so obtained was incubated in its suspending plasma without any additions at 38° and in contact with a gas phase of 5 per cent CO₂-95 per cent air. In 8 hours, a 22 per cent decrease in hemoglobin concentration, as measured spectro-

photometrically, was observed. The differential parasite count indicates that an increase in parasite size occurred during this interval, though it is also obvious from the increased number of degenerate forms that optimum conditions for growth were not present. In other experiments it has been possible to demonstrate that an increase in hematin content of the parasites also occurs during the incubation of such a concentrate of parasitized cells.

We have attempted in several ways to learn something about the mechanism of hematin production by the parasite, but without much success. For example, it has not been possible for us to obtain any spectrophotometric evidence of a breakdown product intermediate between hemoglobin and hematin. Concentrates of parasitized cells such as those used in the

TABLE X

Hemoglobin Destruction in Vitro by Concentrate of Cells Parasitized with Plasmodium knowlesi

Monkey 35-92; April 6, 1944.

Determination	Time of incubation, 38°	
	0 hr.	8 hrs.
Red blood cells, per c.mm.....	30,000	27,000
Parasites, per c.mm.....	26,000	20,000
Rings, %	1	0
Trophozoites, %.....	91	42
Schizonts, %.....	5	29
Segmenters, %.....		3
Degenerate, %.....	3	23
Extracellular, %.....		3
HbO ₂ , log I ₀ /I at λ 576 m μ *.....	0.152	0.118

* Blood diluted 1:6 with distilled water, parasites and cellular debris centrifuged off, and supernatant used for determination in the Beckman spectrophotometer, 1 cm. cell.

experiment depicted in Table X yield, on laking and centrifugation, a solution whose absorption spectrum as measured in the visible range with the Beckman spectrophotometer is identical with that given by oxyhemoglobin from normal monkey red blood cells. Thus the hemoglobin present at any time within a red cell that is host to a parasite is not distinguishable spectroscopically from that present in an unparasitized cell. Either the degradation changes are too subtle to be detected by the means employed, or else degradation of hemoglobin occurs only after it is absorbed by the parasite. The fact that hematin deposition occurs within the parasite favors the latter interpretation, but is, of course, no proof of it.

An attempt has been made to detect an enzymatic degradation of hemoglobin by parasite extracts. Parasites obtained by saponin laking of

parasitized cells were ground and incubated with normal monkey hemoglobin at 38° in the presence of toluene. Though some methemoglobin formation occurred, the total hemoglobin concentration as determined after addition of $\text{Na}_2\text{S}_2\text{O}_3$ showed no appreciable alteration.

A simple procedure for estimating the degree of hematin formation in a sample of blood is to determine the ratio of total alkaline hematin to oxyhemoglobin spectrophotometrically. Since none of the hematin appears to leave the parasitized cell, there is an increase in this ratio as the hemoglobin content diminishes. In Table XI, examples of this procedure as applied to parasitized monkey, human, and duck blood are given. The data presented there were obtained by laking blood in distilled water and in alkaline alcohol (0.04 gm. of KOH in 80 per cent ethyl alcohol). The same dilution was employed in both cases, the final volume being chosen so as to

TABLE XI
Ratio of Hematin to Oxyhemoglobin in Normal and Parasitized Cells

Blood sample	Alkaline hematin HbO ₂	Total cells with pigment	Free hematin Total hematin
		per cent	per cent
Monkey, normal	1 21	0	
“ parasitized, <i>P. knowlesi</i> concen- trate	2 00	56	40
Monkey, parasitized, <i>P. knowlesi</i> concen- trate	2 39	62	49
Human, normal	1 15	0	
“ parasitized, <i>P. vivax</i> concentrate	1 33	37	12
Duck, normal	1.22	0	
“ parasitized, <i>P. lophurae</i>	2.33	34	48

give suitable density readings in the Beckman spectrophotometer. Both samples were centrifuged after standing long enough to permit complete extraction of parasite hematin by the alkaline alcohol; 15 minutes usually suffice. The completeness of extraction may be roughly checked by the whiteness of the precipitate obtained after centrifuging. The $\log I_0/I$ values of the supernatant were then read in a cell with a 1 cm. light path at the following wave-lengths: 500, 510, 520, 540, 550, 560, 575, 586, 593, 600, 620 μ . The resulting density readings were then totaled for each solution and their ratio determined. It was found that this procedure gave more reproducible results than the employment of readings at only one or two wave-lengths. The ratios for different normal blood samples from any one species by this technique agree within ± 2 per cent. The ratio for normal human blood is slightly lower than for monkey or duck blood. From the ratio of alkaline hematin to oxyhemoglobin determined by this

method for parasitized blood, it is possible to calculate the per cent of total hematin which exists as free hematin by use of the following formula.

$$\frac{(\text{Parasitized blood ratio}) - (\text{normal blood ratio})}{\text{Parasitized blood ratio}} \times 100 = \% \text{ of total hematin free}$$

Values calculated by this formula are given in the last column of Table XI. For comparison, there is given an estimate made microscopically by one of us (W. O. C.) of the per cent of total cells in which pigment deposition was visible.

DISCUSSION

In considering the results presented here, it should be emphasized that they do not represent the maximum changes that can be expected to occur within a parasitized red blood cell. The maximum differences in chemical and metabolic changes would undoubtedly be found by comparing normal red cells with cells containing only full grown parasites; *i.e.*, segmenters which are just ready to release their merozoites. In no blood sample studied here have such conditions prevailed, nor have we attempted to extrapolate our values to a theoretical maximum achievable mass of parasite material. Thus the values for the parasitized cell to be dealt with here must be considered only as approaching the maximum to be expected, and might fall well short of it in some cases.

One of the most pronounced changes encountered in comparing the parasitized cell with the normal is the increase in nucleic acid phosphorus. Cytological observation has long indicated that material with the staining properties of nucleic acid accumulates within the parasite during its growth. The identification of a portion of this as desoxyribose nucleic acid has been described in Paper II of this series (4). It is now possible to make some quantitative estimates of the total nucleic acid fraction in the parasitized cell and to compare it with other nucleated cells. From the data of Table I, it may be calculated that 5×10^{12} parasitized cells with the differential count given in the third column contain 17.6 mM more of nucleic acid P than do a similar number of normal red cells. This corresponds to 17.6×31 mg. or 545.6 mg. of nucleic acid P. To compare this value with values given in the literature for other tissues, it is necessary to express it in terms of gm. of wet weight of cells. The volume occupied by 5×10^{12} cells may be assumed to be 450 cc. Since, as shown in Paper III of this series (5), the parasitized cell will remain suspended in an albumin solution with a density of 1.07, this figure may be used as the specific gravity of the parasitized cell. Thus, 5×10^{12} parasitized cells will have a wet weight of 450×1.07 gm. or 481 gm. The amount of nucleic acid P per 100 gm. of wet weight of parasitized cells is thus 545.6 mg. divided by 481, or 114 mg.

The values given by Schmidt and Thannhauser (17) for total mg. of nucleic acid P per 100 gm. of wet weight of tissue range from 47 for rat brain to 182 to 238 for calf thymus, with rat liver intermediate, showing values of 113 to 125. The nucleic acid P content of red cells parasitized with *Plasmodium knowlesi* thus falls within the range encountered in mammalian tissues. Since nucleic acids contain roughly 10 per cent phosphorus, the total nucleic acid in the 5×10^{12} parasitized cells considered here is 10×545.6 mg., or 5456 mg.

It would appear from data obtained on parasites grown *in vitro* that the parasite does not need to be supplied with nucleic acids as such. Presumably, as indicated by the data presented here, it can synthesize *in vitro* its own nucleic acids from simpler compounds. Whether, however, the parasite can synthesize, for example, all its own purines or pyrimidines which it incorporates into nucleic acid cannot be answered definitely. The available evidence would suggest that such a synthesis may be possible. The case of adenine may be taken as a specific example. In the parasitized blood samples discussed in the preceding paragraph, an increase of 17.6 mm of nucleic acid P occurred per 5×10^{12} parasitized cells. If one-fourth of this phosphorus is equal to the adenine in the basic tetranucleotide, then an increase of 4.4 mm of adenine has occurred in those parasitized cells. In addition, there has occurred in these same cells an increase of 1.8 mm in the 15 minute-hydrolyzable P, which, if accepted as coming from adenosine triphosphate, represents a corresponding increase in adenine. A total increase of $4.4 + 1.8$ or 6.2 mm of adenine has thus occurred. The amount contributed by the increase in flavin-adenine dinucleotide may be neglected. Now 6.2 mm of adenine equal an increase of 837 mg. of adenine per 5×10^{12} parasitized cells. On this basis, 1 cc. of whole blood containing the normal value of 5×10^9 unparasitized cells should increase its adenine content by 837 γ when 100 per cent of the cells were parasitized, or 8.37 γ for each 1 per cent absolute change in parasitized cell count. Now *in vitro* by the rocker dilution technique (7), increases in absolute count from 2 to 8 per cent parasitization in 24 hours have been regularly obtained. Thus for 6 per cent of the cells to become parasitized, if changes *in vitro* are assumed to be similar to those *in vivo*, it should require 50.2 γ of adenine per 1 cc. of blood. The 3 cc. of media supplied per cc. of blood in these experiments contain, however, only 0.75 γ of adenine. The amount supplied in the standard medium thus falls far short of the amount calculated in this way to be needed and suggests that the parasite is able to synthesize adenine. In unpublished experiments, no effect on growth *in vitro* of the parasite was observed when the adenine and the other purine and pyrimidine content of the medium was increased 10-fold over the standard, though it is now obvious that even greater increases should be tried. We have reported (1),

however, a cultivation experiment by the perfusion technique which showed that the complete absence of all purine and pyrimidine from the nutrient medium resulted in poor growth. The question of the synthesis by the malarial parasite of its purines and pyrimidines is obviously not answered by these calculations, but the magnitude of its requirements for these components is certainly better defined.

The specific gravity of the parasitized cell is less than that of the normal cell. This fact has been known for some time, and we have made use of it to effect the separation of parasitized and normal cells (5, 7). This change can be in part explained by the increase in lipide material that occurs when the normal cell becomes parasitized. Increases of 400 per cent in both phospholipide P and fatty acid have been reported in Tables I to III. Morrison and Jeskey (14) report increases of 550 per cent in total lipides in cells parasitized by *Plasmodium knowlesi*. Their values include non-saponifiable matter, mainly cholesterol, which they find averages 25 per cent of the total lipides. Our findings thus appear to be in good agreement with theirs, though a comparison of actual values is not possible because these authors give no such data for the parasitized cell in their preliminary report. However, Morrison and Jeskey state that 28.8 per cent of the total solids of isolated parasites is lipide material. They also report that the fatty acids are largely C_{18} compounds. Using our data and assuming that the fatty acids are C_{18} compounds, that the phospholipide fraction is lecithin, and the water content of the parasitized cell is 75 per cent, we have calculated that the maximum saponifiable lipide content in the parasitized cells studied here is 6.5 per cent of the total dry weight. Applying a correction for non-saponifiable material yields results which are approximately one-third of those reported by Morrison and Jeskey for the parasite itself. This difference must be in part attributable to the fact that in dealing with analyses on parasitized cell samples we are not employing 100 per cent parasite material.

The results reported here indicate that parasites growing and multiplying *in vitro* are not entirely comparable in their metabolic and chemical patterns to those grown *in vivo*. Differences such as those that have been observed in oxygen consumption and fatty acid-phospholipide ratios furnish hints as to improvements in the basic culture medium that need further exploration. Any improvement that can be so achieved should also facilitate the culture of the more fastidious human malarial parasites which as yet respond less satisfactorily than *Plasmodium knowlesi* to our cultivation techniques.

SUMMARY

1. Certain metabolic and chemical changes occurring in the red blood cells of monkeys as a result of their invasion, either *in vivo* or *in vitro*, by

the malarial parasite, *Plasmodium knowlesi*, have been followed. The magnitude of the increases that occur is shown to be dependent on both the size and age of the parasites and the total number of parasites present.

2. In the studies reported here, the invasion of the red blood cell by the parasite *in vivo* results in a 25- to 75-fold increase in the rate of oxygen and glucose consumption. The molar ratio of the rate of utilization of oxygen to glucose is approximately unity, indicating that an amount of glucose equivalent to only one-sixth of the total that disappears is completely oxidized. From 54 to 82 per cent of the glucose that disappears can be accounted for as lactate.

3. The parasitized cell content of fatty acids is 4- to 5-fold, of total phosphorus 2- to 4-fold, of 15 minute-hydrolyzable P 2- to 4-fold, of phospholipide P 2- to 4-fold, of nucleic acid P 10- to 20-fold, of flavin-adenine dinucleotide 6- to 15-fold that of the normal red blood cell.

4. In the case of parasites growing and multiplying within red cells cultivated *in vitro*, increases in these same values may be observed as the parasite number increases. The changes are not, however, as consistent nor of the same magnitude as for parasites grown *in vivo*. This is particularly striking in the case of oxygen consumption, for which no increase in rate is usually observed. A deficiency of unknown essential nutrients in the culture medium is suggested by these results.

5. The molar ratio of fatty acids to phospholipide P in normal monkey cells is on the average 2.46. In parasitized cells, it averages 2.64. It is concluded that little neutral fat exists in either normal or parasitized cells.

6. Normal monkey and human red blood cells are able to synthesize flavin-adenine dinucleotide *in vitro* from the components of the culture medium. Increases observed in the content of this coenzyme in parasitized cells during growth and multiplication of the parasite *in vitro* cannot be attributed, therefore, solely, if at all, to the parasite itself. It is pointed out that the relation of this observation to the action of atabrine deserves investigation.

7. The conversion of hemoglobin to hematin by the parasitized cell has been followed quantitatively both *in vivo* and *in vitro*. The total hematin content of the parasitized cell remains constant during the growth of the parasite, though a nearly complete conversion of hemoglobin to hematin may occur during the parasites' full development. No spectroscopic evidence for any intermediate in this conversion could be obtained.

8. A simple procedure for estimating the degree of hematin formation in a sample of parasitized blood is to determine spectroscopically the ratio of total alkaline hematin to oxyhemoglobin. Representative examples of values on human, duck, and monkey blood are given.

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ENZYMATIC HYDROLYSIS OF 2,4-DIKETO ACIDS

BY ALTON MEISTER AND JESSE P. GREENSTEIN

(From the National Cancer Institute, National Institute of Health, Bethesda, Maryland)

(Received for publication, May 13, 1948)

In the course of studies on the desamidation of glutamine in rat liver extracts marked acceleration of this reaction was noted in the presence of 2,4-diketovaleric acid (acetopyruvic acid).¹ Recent work indicates that pyruvic acid and certain other α -keto acids increase the desamidation of glutamine in aqueous extracts of normal and neoplastic rat liver (1-3). Investigation of the mechanism of the acceleration of glutamine desamidation by 2,4-diketovaleric acid revealed that this effect was actually due to pyruvic acid formed by hydrolysis of the diketo acid and that 2,4-diketovaleric acid itself was apparently inactive in the glutamine system (4). This finding led to experiments on other 2,4-diketo acids. The present studies demonstrate the existence of a hitherto unrecognized enzymatic reaction occurring in extracts of liver and kidney, whereby a wide variety of 2,4-diketo acids (acylpyruvic acids) are hydrolyzed to yield pyruvic acid and the corresponding fatty acid.

EXPERIMENTAL

Preparation of Disodium Salts of 2,4-Diketo Acids—The general procedure of Claisen and Stylos (5) was employed for the synthesis of 2,4-diketo acid esters. Ethyl oxalate was condensed with the appropriate methyl ketone in the presence of sodium ethoxide to yield the ethyl ester of the corresponding 2,4-diketo acid. The methyl ketones were obtained from the Eastman Kodak Company and redistilled before use. Methyl *n*-heptyl ketone was prepared by the acetoacetic ester synthesis (6). The preparation of free 2,4-diketo acids and the corresponding ethyl and methyl esters has been adequately described (see references, Table I). However, we have found it possible to prepare the disodium salts of the 2,4-diketo acids which, in contrast to the free diketo acids, are stable over long periods.

Disodium salts of the normal aliphatic 2,4-diketo acids and the aromatic diketo acids were prepared as follows: The ester was added to 0.5 volume of acetone and the mixture gently shaken with 2 equivalents of 5 *N* sodium hydroxide. Saponification of the ester was accompanied by some evolu-

¹ The authors are indebted to Dr. Albert L. Lehninger for suggesting that the effect of this compound on glutamine desamidation be studied.

tion of heat. The disodium salt of the diketo acid was then precipitated from the clear solution by addition of a large volume of alcohol. The disodium salt was washed with alcohol and ether and dried *in vacuo*. Acetone may be omitted in the preparation of disodium 2,4-diketovalerate but it facilitates saponification of the other esters. The disodium salts may be reprecipitated from water-alcohol mixtures. Ethyl 2,4-diketo-5-methyl hexanoate, ethyl 2,4-diketo-6-methyl heptanoate, and ethyl 2,4-diketo-5,5-dimethyl hexanoate were saponified as follows: The esters were shaken with 2 equivalents of 0.5 N sodium hydroxide at 5° for 2 to 4 hours. A precipitate, probably of the sodium salt of the ester, formed initially on addition of the alkali and redissolved on prolonged shaking. The disodium salt of the acid was then precipitated by addition of acetone. Attempts to saponify these esters by the former procedure invariably resulted in salts which gave sodium analyses several per cent higher than the theoretical values.

The compounds prepared in the present study are listed in Table I, together with analytical data for the ethyl esters and disodium salts. The 2,4-diketo acids may be prepared from the corresponding disodium salts. Free 2,4-diketovaleric acid was prepared as follows: An acidified solution of the disodium salt was extracted several times with ether. The solution was dried over anhydrous sodium sulfate and evaporated until crystals appeared. The acid was recrystallized from carbon tetrachloride; m.p. 98° (uncorrected).

Analysis— $C_5H_8O_4$. Calculated, C 46.16, H 4.65; found, C 46.17, H 4.59

The following 2,4-diketo acids were precipitated from aqueous solutions of the disodium salts by acidification with N hydrochloric acid and subsequently recrystallized from ether and petroleum ether.

2,4-Diketoundecylic acid, $C_{11}H_{18}O_4$; m.p. 50° (uncorrected)

Calculated, C 61.65, H 8.47; found, C 61.43, H 7.97

2,4-Diketo-4-phenylbutyric acid, $C_{10}H_{16}O_4$; m.p. 157° (uncorrected)

Calculated, C 62.49, H 4.20; found, C 62.52, H 3.97

2,4-Diketo-4-(4-methylphenyl)-butyric acid, $C_{11}H_{18}O_4$; m.p. 142° (uncorrected)

Calculated, C 64.07, H 4.89; found, C 63.89, H 5.03

The enzymatic and spectral properties of these compounds were identical with those of the corresponding disodium salts.

Spectrophotometric Characterization of 2,4-Diketo Acids and Esters—The 2,4-diketo acids and ethyl esters possess characteristic absorption in the ultraviolet region which is associated with their enolic structure. Ethyl 2,4-diketovalerate and the ethyl esters of the other aliphatic 2,4-diketo acids studied possess absorption maxima at 2900 Å. Ethyl 2,4-diketo-4-

phenyl butyrate and ethyl 2,4-diketo-4-(4-methylphenyl) butyrate exhibit a broad maximum absorption between 3050 and 3150 Å and somewhat greater molar extinction coefficients. The maximum molar extinction coefficients for the various 2,4-diketo acid esters, determined with the

TABLE I
Characterization of 2,4-Diketo Acid Derivatives

Corresponding 2,4-diketo acid	Ethyl ester					Disodium salt		Bibliographic reference No.
	Carbon		Hydrogen		Maximum molar extinction coefficient	Sodium		
	Calcu- lated	Found	Calcu- lated	Found		Calcu- lated	Found	
	per cent	per cent	per cent	per cent		per cent	per cent	
<i>n</i> -Valeric	53.18	53.09	6.38	6.21	9,010* (1.40 × 10 ⁻⁴ M)	26.42	26.23	(7-10)
<i>n</i> -Hexanoic *	55.80	55.63	7.02	7.04	8,500* (1.25 × 10 ⁻⁴ M)	24.45	23.54	(9-12)
<i>n</i> -Heptanoic	58.05	57.78	7.58	7.40	8,950* (1.43 × 10 ⁻⁴ M)	22.76	21.97	(9, 10, 13)
<i>n</i> -Octanoic	59.99	60.07	8.06	7.77	9,100* (1.42 × 10 ⁻⁴ M)	21.27	21.42	(10, 14)
<i>n</i> -Nonanoic	61.65	62.09	8.47	8.49	8,760* (1.05 × 10 ⁻⁴ M)	19.98	20.08	(10)
<i>n</i> -Capric	63.13	63.15	8.83	8.81	8,850* (1.23 × 10 ⁻⁴ M)	18.83	18.92	(10)
<i>n</i> -Undecylic	64.44	64.45	9.15	9.18	8,400* (1.89 × 10 ⁻⁴ M)	17.81	17.92	(10)
5-Methyl- <i>n</i> -hexanoic	58.05	57.76	7.58	7.56	8,970* (1.57 × 10 ⁻⁴ M)	22.76	22.28	(13)
6-Methyl- <i>n</i> -hep- tanoic	59.99	59.87	8.06	7.90	9,780* (1.53 × 10 ⁻⁴ M)	21.27	20.79	(9, 15)
5,5-Dimethyl- <i>n</i> - hexanoic	59.99	60.00	8.06	8.04	9,460* (1.34 × 10 ⁻⁴ M)	21.27	21.26	(9, 16)
4-Phenylbutyric	65.45	65.75	5.49	5.48	12,900† (1.14 × 10 ⁻⁴ M)	19.48	19.05	(17)
4-(4-Methylphenyl)- butyric	66.67	66.87	6.03	6.38	14,800† (1.02 × 10 ⁻⁴ M)	18.38	18.55	

* At 2900 Å in ether.

† At 3100 Å in ether.

Beckman model DU spectrophotometer, are given in Table I. The relationship between degree of enolization and ultraviolet absorption was demonstrated by a study of the absorption of 2,4-diketovaleric acid at different values of pH. As the free diketo acid is converted to the enolic

form by addition of base, the peak absorption shifts from 2850 to 2950 Å, and the maximum molar extinction coefficient increases from 3030 to 18,400 (Fig. 1). The changes in absorption due to addition of acid or base are completely and instantaneously reversible. When the change in absorption is plotted as a function of pH, points are obtained which closely fit theoretical titration curves based upon pK values of 2.5 and 7.8 (Fig. 2). The corresponding values obtained by acidometric titration are 2.61 and 7.85.

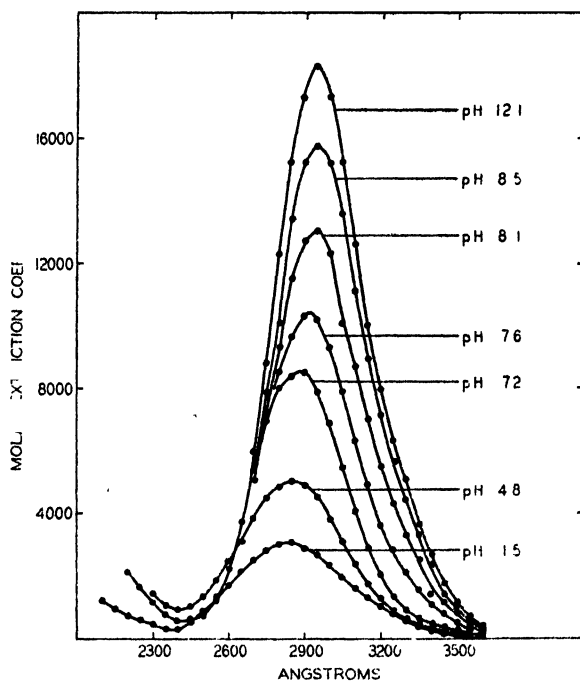


FIG. 1. Ultraviolet absorption curves of 2,4-diketovaleric acid at various values of pH. Concentration, 0.726×10^{-4} M.

Other Materials—Acetylacetone, an Eastman product, was redistilled before use. The glutamine was a gift of the American Cyanamide Company. Approximately 200 male and female rats of the Buffalo strain, weighing 150 to 350 gm., seven Strain A mice, five albino rabbits, and four cats were used in the present study. Primary rat hepatomas were induced by feeding *p*-dimethylaminoazobenzene. The animals were fed *ad libitum* except in the case of the cats, which were fasted for 3 days before use.

Analytical Methods—Pyruvate was determined by the method of Lu (18) with minor modifications. The aniline citrate method of Edson (19) was

employed for the detection of acetoacetate. Acetate was determined as described by Lipmann (20) and octanoate by the new procedure of Lehninger and Smith (21). Ammonia was determined by nesslerization after aeration into sulfuric acid traps. Protein nitrogen analyses were made by the usual Kjeldahl technique. The Beckman pH meters, models G and H, were employed for the measurement of pH.

Enzymatic Studies—The animals were killed by decapitation or by a blow on the head, followed by exsanguination. Tissue extracts were prepared by grinding the fresh tissue with 3 volumes of water in a glass mortar, followed by light centrifugation to remove the larger particles. Studies in

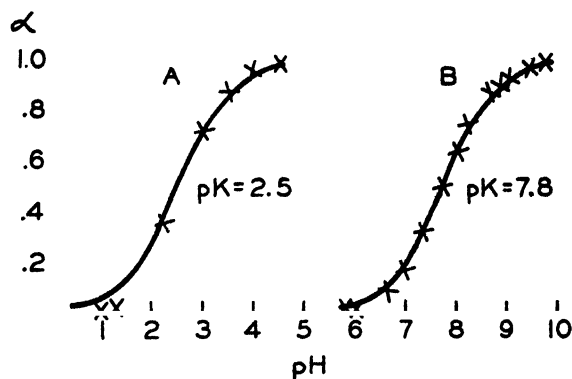


FIG. 2. Spectrophotometric titration curves of 2,4-diketovaleric acid. Theoretical curves for $\text{pH} = \text{pK} + \log (\alpha/1-\alpha)$. $\alpha = \Delta D/D$, where D represents the total change in absorption and ΔD the increment for a given change in pH. The data were obtained at 2850 and 2950 Å for Curves A and B, respectively. Concentration, 0.690×10^{-4} M. Absorption was determined in 0.1 M glycine hydrochloride, acetate, phosphate, and borate buffers for the pH ranges 1.0 to 3.7, 3.8 to 4.9, 5.2 to 7.8, and 7.9 to 10.0, respectively.

which extracts were prepared with the Waring blender or Potter-Elvehjem homogenizer yielded similar data when calculated on a tissue nitrogen basis. All experiments were carried out in a constant temperature water bath at 37°. The disodium salts of the 2,4-diketo acids were dissolved in veronal or borate buffer and the pH adjusted, when necessary, to the desired value by the addition of dilute hydrochloric acid. No significant difference was noted between data obtained with borate buffers and the values found with veronal. Usually 1 cc. of extract was incubated with 1 cc. of buffer containing the substrate. Following incubation, 5 cc. of 5 per cent trichloroacetic acid were added and the clear filtrate analyzed for pyruvate. Controls with substrate alone and with extract alone were employed.

Conventional Warburg vessels were employed for the experiments on

liver slices. After equilibration with 100 per cent oxygen or nitrogen, liver slices weighing 200 mg. were shaken with 2.3 cc. of a solution containing 0.1 M phosphate buffer (pH 7.4), 0.154 M sodium chloride, 0.02 M ammonium chloride, and 0.01 M disodium 2,4-diketovalerate (23 micromoles). At the end of 30 minutes the vessel contents were treated with trichloroacetic acid and analyzed for pyruvate by the procedure of Elgart and Nelson (22). Control vessels were employed in which tissue or diketovalerate was omitted.

Experiments on Glutamine Desamidation—The accelerating effect of 2,4-diketovalerate and pyruvate on the desamidation of glutamine in aqueous extracts of normal liver is demonstrated in Table II. Although pyruvate also increased the desamidation of glutamine in extracts of

TABLE II

Effect of 2,4-Diketovaleric and Pyruvic Acids on Desamidation of Glutamine

1 cc. of tissue extract was incubated for 4 hours with 1 cc. of 0.15 M veronal buffer at pH 7.0, 1 cc. of 0.014 M glutamine (14 micromoles), and 1 cc. of 0.028 M sodium pyruvate or disodium 2,4-diketovalerate (28 micromoles) or water.

Rat tissue	Keto acid	Ammonia formed <i>micromoles</i>	Per cent increase
Normal liver.....	None	3.98	
“ “	Pyruvic	10.8	171
“ “	Diketovaleric	10.7	169
Primary hepatoma ..	None	2.43	
“ “	Pyruvic	3.72	53.1
“ “	Diketovaleric	2.41	0

hepatoma, the addition of diketovalerate to such extracts did not affect the rate of ammonia liberation from glutamine.

Conversion of 2,4-Diketovalerate to Pyruvate and Acetate—Investigation of the mechanism of the acceleration of glutamine desamidation by diketovalerate revealed that this compound was hydrolyzed to yield nearly equivalent amounts of pyruvate by rat liver extracts (Fig. 3). Diketovalerate is stable under these conditions in the absence of liver extract, and no pyruvate was formed when diketovalerate was incubated with boiled liver extract. The reaction occurred equally well in the absence of oxygen. The pyruvate formed was identified by isolation as the 2,4-dinitrophenylhydrazone which was recrystallized from ethyl acetate. The melting points and spectra of the isolated compound and an authentic sample were identical (m.p. 214°; mixed m.p. 214°; uncorrected). No detectable acetoacetate formation occurred. Acetate production was demonstrated by the following experiment. 10 cc. of liver extract were incubated with 10 cc. of

0.15 M borate buffer at pH 7.3 containing 0.678 mM of diketovalerate for 10 minutes, and the mixture was treated with phosphotungstic acid. Analysis of the protein-free filtrate revealed the appearance of 0.182 and 0.155 mM of pyruvate and acetate, respectively.

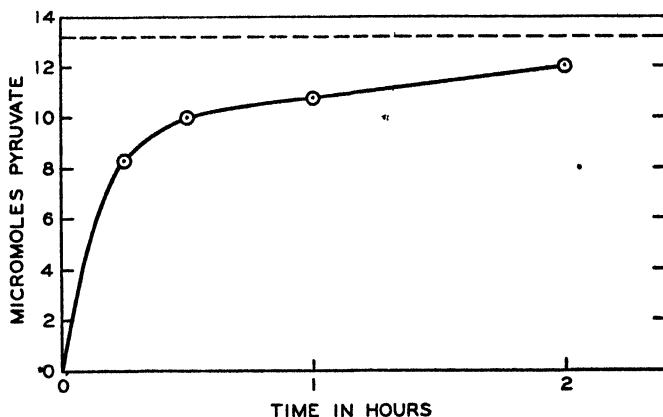


FIG. 3. Hydrolysis of 2,4-diketovaleric acid by rat liver extract. The data were obtained with 1 cc. of extract and 1 cc. of 0.15 M veronal buffer at pH 7.3 containing 13.2 micromoles of substrate.

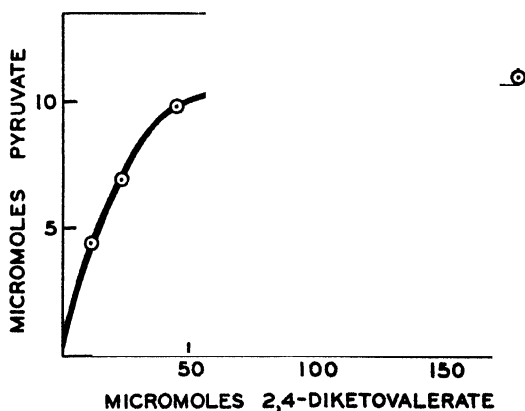


FIG. 4. Effect of 2,4-diketovalerate concentration on hydrolysis. The data were obtained with 1 cc. of rat liver extract (5.23 mg. of N per cc.) and 1 cc. of 0.15 M veronal buffer at pH 7.3 containing substrate. Incubated for 10 minutes.

The effect of diketovalerate concentration on the rate of pyruvate appearance in rat liver extract is illustrated in Fig. 4. Maximum hydrolysis occurs with diketovalerate concentrations of 50 micromoles or greater under these conditions. The relationship between liver extract concentra-

tion and hydrolysis is linear below a concentration of extract equivalent to 2.5 mg. of nitrogen per cc. (Fig. 5).

The hydrolysis of 2,4-diketovalerate by several animal tissues was investigated (Table III). Of the tissues examined, only liver and kidney hydrolyzed diketovalerate at an appreciable rate. It is of interest that rat hepatoma has little hydrolytic activity. Since diketovalerate does not accelerate glutamine desamidation in extracts of rat hepatoma, although pyruvate does, it would appear that 2,4-diketovalerate itself is not active in the glutamine system. This conclusion is based on the assumption that

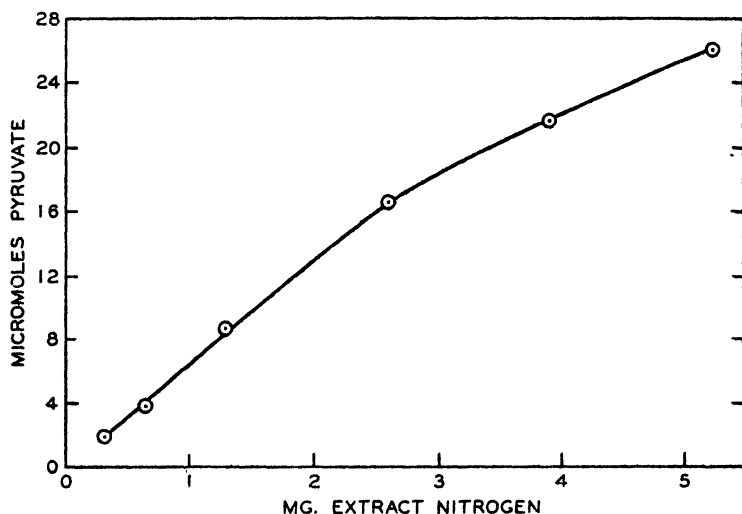


FIG. 5. Relation between liver extract concentration and hydrolysis. The data were obtained with 1 cc. of rat liver extract and 1 cc. of 0.15 M veronal buffer at pH 7.3 containing 68 micromoles of 2,4-diketovalerate. Incubated for 20 minutes.

the enzymes of hepatoma and normal liver responsible for this reaction are the same.

Formation of pyruvate was also observed in experiments in which liver slices were shaken with diketovalerate. Under the conditions described above, 4.63 and 5.90 micromoles of pyruvate appeared in 30 minutes aerobically and anaerobically, respectively.

Hydrolysis of Other 2,4-Diketo Acids—All the 2,4-diketo acids of the present series were hydrolyzed by extracts of liver and kidney, as determined by the appearance of pyruvate. The relationship between the rates of hydrolysis of the aliphatic 2,4-diketo acids and the length of the carbon chain, under the conditions employed, is demonstrated in Table IV. The relative rates of hydrolysis were approximately the same when 23 micromoles of substrate were employed. The aromatic diketo acids were

hydrolyzed at considerably slower rates than those for the aliphatic group. The activity ratios for the several substrates in liver and kidney extracts are about the same.

The pH-activity curves for the hydrolysis of the normal 2,4-diketo acids containing 5 to 11 carbon atoms and of 2,4-diketo-4-phenyl butyrate by liver extract were determined. Representative data are given in Fig. 6.

TABLE III

Hydrolysis of 2,4-Diketovaleate by Various Animal Tissues

Data obtained with 1 cc. of tissue extract and 1 cc. of 0.15 M veronal buffer at pH 7.3, containing 68 micromoles of disodium 2,4-diketovaleate.

Tissue		Micromoles pyruvate per mg. tissue N		
		15 min.	30 min.	120 min.
Rat	Liver	3.46	5.61	8.25
	Kidney	2.04	4.13	6.56
	Pancreas		0.449	1.20
	Lung		0.238	1.03
	Brain		0.239	0.964
	Spleen		0.241	0.964
	Intestine		0.204	0.744
	Stomach		0.175	0.677
	Cardiac muscle		0.153	0.510
	Testis			0.360
	Skeletal muscle			0.301
	Primary hepatoma		0.272	0.473
Mouse	Liver	5.04	6.46	9.63
	Kidney	2.83	4.36	6.90
	Skeletal muscle			0.565
Rabbit	Liver	1.43	2.14	3.20
	Kidney	0.645	1.17	2.11
	Skeletal muscle			0.290
Cat	Liver	2.90	3.98	6.65
	Kidney	1.18	1.71	3.10
	Skeletal muscle		0.211	0.475

The pH optimum for the hydrolysis of diketovaleate in rat kidney extract was identical with that observed for liver. The optimal pH ranges for the hydrolysis of 2,4-diketohexanoate, 2,4-diketononanoate, and 2,4-diketocaproate were 7.5 to 7.8, 8.0 to 8.6, and 8.0 to 8.5, respectively. Identical pH optima were observed in veronal buffers and with lower substrate concentrations.

Hydrolysis of 2,4-diketoundecylate would be expected to yield equimolar amounts of pyruvate and octanoate. In Fig. 7 are given the values of octanoate and pyruvate formed when 2,4-diketoundecylate was incubated

TABLE IV

Hydrolysis of 2,4-Diketo Acids by Extracts of Rat Liver and Kidney

Experiments carried out with 1 cc. of tissue extract and 1 cc. of 0.15 M borate buffer containing 68 micromoles of substrate. Aliphatic substrates were incubated for 10 and 15 minutes with liver and kidney extracts, respectively. Aromatic substrates were incubated for 60 minutes.

Substrate	pH	Micromoles pyruvate per mg. extract N per hr.	
		Liver	Kidney
2,4-Diketovalerate	7.3	15.0	7.64
2,4-Diketoheptanoate.....	7.6	23.7	14.7
2,4-Diketoheptanoate.....	7.6	5.54	3.14
2,4-Diketooctanoate	8.3	4.22	2.15
2,4-Diketononanoate.....	8.3	10.1	5.31
2,4-Diketocaprato.....	8.3	20.2	9.61
2,4-Diketoundecylate	8.3	6.63	2.97
2,4-Diketo-5-methyl hexanoate.....	7.8	14.9	8.90
2,4-Diketo-6-methyl heptanoate	7.8	2.89	1.50
2,4-Diketo-5,5-dimethyl hexanoate	7.8	6.15	3.65
2,4-Diketo-4 phenyl butyrate.....	7.8	0.354	0.185
2,4-Diketo-4-(4-methylphenyl) butyrate.....	7.8	0.309	0.197

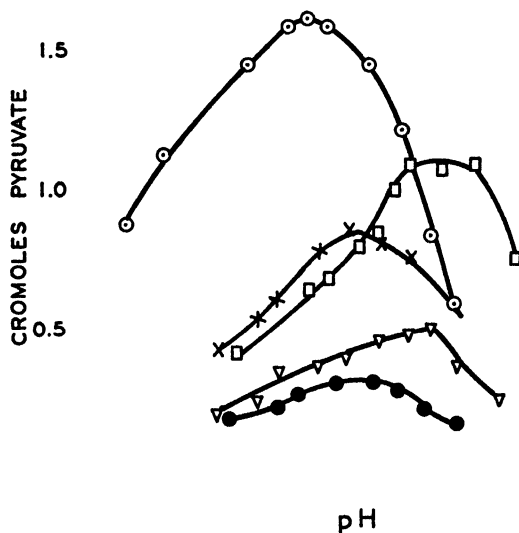


FIG. 6 pH-activity curves for the hydrolysis of the disodium salts of several 2,4-diketo acids by rat liver extract. ○ = 2,4-diketovalerate; × = 2,4-diketoheptanoate; ▽ = 2,4-diketooctanoate; □ = 2,4-diketoundecylate; ● = 2,4-diketo-4-phenyl butyrate. The values were obtained with 1 cc. of extract and 3 cc. of 0.1 M borate buffer containing 68 micromoles of substrate. Incubated for 10 minutes with the aliphatic substrates and for 60 minutes with 2,4-diketo-4-phenyl butyrate.

with liver extract. Within experimental error the data indicate the appearance of equivalent quantities of these products, the reaction therefore being analogous to the hydrolysis of 2,4-diketovalerate.

Hydrolysis of 2,4-diketohexanoate to pyruvate was also demonstrated under conditions similar to those of Breusch and Ulusoy (23). 3 gm. of minced cat liver were shaken with 111 micromoles of disodium 2,4-diketohexanoate in 7 cc. of 0.154 M sodium chloride at 37° in a Warburg vessel for 45 minutes. Analysis of the vessel contents revealed the appearance of 65.4 micromoles of pyruvate, representing hydrolysis of 59 per cent of this substrate.

Preparation of Partially Purified Enzyme from Liver—By means of low temperature alcohol fractionation, preparations were obtained from rat

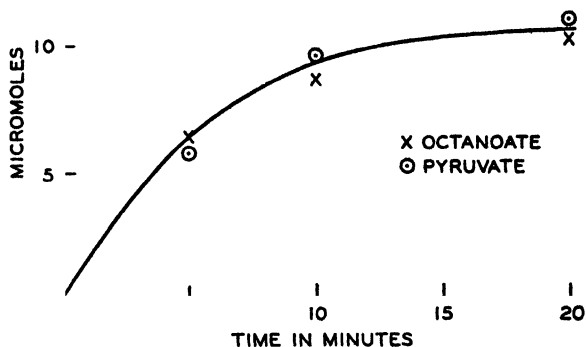


FIG. 7. Hydrolysis of 2,4-diketoundecylate by rat liver extract. The data were obtained with 1 cc. of extract and 1 cc. of 0.15 M borate buffer at pH 8.3 containing 68 micromoles of substrate.

liver in which the hydrolytic activity towards 2,4-diketo acids was concentrated about 9- or 10-fold. The following procedure was employed. Fresh rat liver was homogenized in a Waring blender with 3 volumes of distilled water and the pH of the homogenate adjusted to 4.9 with 0.2 M acetic acid. Ethanol (50 per cent) was added at -5° to a final concentration of 15 per cent. After standing for 12 hours the mixture was centrifuged at -3° . Most of the protein was precipitated, leaving 75 to 80 per cent of the activity in solution. The supernatant was adjusted to pH 6.5 with 0.1 M sodium hydroxide, brought to a final concentration of 50 per cent ethanol, and allowed to stand at -5° for 12 hours. The precipitate was collected by centrifugation, dissolved in water, and the small insoluble residue removed by centrifugation. This clear solution was stored at 5° , and retained its initial activity for several weeks. The activity values with respect to 2,4-diketovalerate for two such preparations were 23.7 and 27.8 micromoles of pyruvate per mg. of nitrogen per

10 minutes. The corresponding values for the initial homogenates were 2.78 and 2.88. The activity ratios for the hydrolysis of the other diketo acids by this preparation were approximately the same as those of rat liver extract.

Spectrophotometric Determination of Enzyme Activity—As described above, the aliphatic 2,4-diketo acids possess a characteristic absorption band at 2950 Å at alkaline pH. This property may be utilized in following the hydrolysis of 2,4-diketovaleate. When this substrate is incubated with liver extract, a progressive decrease in absorption occurs at all wave-lengths. The products of hydrolysis exhibit negligible absorption at 2950 Å and the

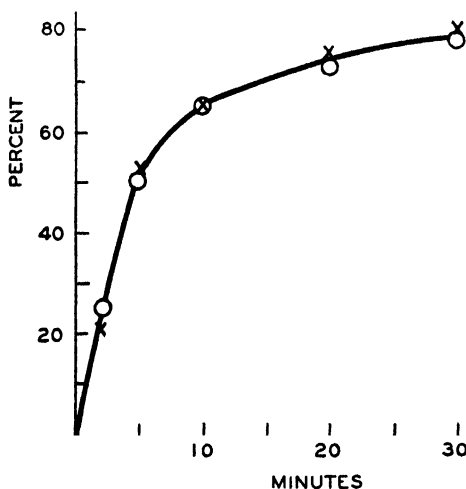


FIG. 8. Hydrolysis of 2,4-diketovaleate by a partially purified liver preparation as determined by chemical and spectrophotometric methods. X = per cent of theoretical pyruvate formed; O = per cent decrease in absorption at 2950 Å. Experimental details are given in the text

absorption due to 2,4-diketovaleate at this wave-length is directly proportional to concentration. The following procedure was employed in following the hydrolysis of 2,4-diketovaleate. 0.2 cc. of 0.15 M borate buffer at pH 7.3, containing 0.688 micromole of diketovaleate, is incubated with 0.2 cc. of diluted extract or the liver preparation described above. Following incubation, 10 cc. of 0.1 M sodium hydroxide are added and the diluted mixture read in the Beckman model DU spectrophotometer against an extract blank at 2950 Å. Initial density readings of 1.22 are obtained under these conditions. Controls with substrate alone show no decrease in absorption. The data given in Fig. 8 illustrate the application of this method to the hydrolysis of 2,4-diketovaleate by the partially purified liver preparation. Good agreement between

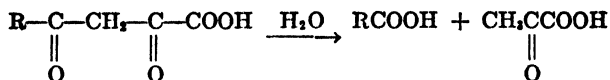
pyruvate formation and the disappearance of absorption was observed. The rate of hydrolysis is determined from the linear part of the curve. It is probable that this technique could readily be applied to other diket acids.

In order to characterize further the substrate specificity requirements of this enzymatic reaction, acetylacetone was subjected to enzymatic study by the spectrophotometric method. This diketone has an absorption curve similar to that of an aliphatic 2,4-diketo acid with a band at 2950 Å at pH 10. After incubation of acetylacetone with the partially purified liver preparation for 30 minutes at values of pH from 6.8 to 8.4, the greatest decrease in absorption was 5 per cent. It would appear, therefore, that replacement of the carboxyl group of 2,4-diketovaleic acid by a methyl group renders the compound much less susceptible to enzymatic attack.

DISCUSSION

The 2,4-diketo acids have received relatively little attention from biochemical workers. In 1937, Krebs and Johnson reported the formation of acetoacetic and β -hydroxybutyric acids from 2,4-diketovaleate in liver slices (24). These authors postulated a condensation of acetic and pyruvic acids to yield 2,4-diketovaleic acid, followed by decarboxylation of the latter, resulting in ketone body formation. However, recent experiments with C^{18} appear to rule out 2,4-diketovaleate as an intermediate in the formation of acetoacetate from pyruvate (25). In studies on intact animals, Lehninger found that fasting ketosis was greatly augmented by the administration of 2,4-diketovaleate. However, this compound also appeared to protect against death by insulin hypoglycemia. Furthermore, a small rise in the blood pyruvate level was noted after injection of 2,4-diketovaleate (26). In contrast to the latter compound, 2,4-diketo-octanoate was only slightly absorbed from the gastrointestinal tract of rats and did not increase the oxygen consumption of rat tissue slices (14).

The present experiments show that pyruvate is formed from 2,4-diketovaleic acid and other acylpyruvic acids in extracts of liver and kidney. Acetate and pyruvate are formed from 2,4-diketovaleate in about the same molar ratio. The reaction occurs aerobically and anaerobically, and without detectable acetoacetate formation. Furthermore, pyruvate was formed from 2,4-diketovaleate in experiments with liver slices under conditions similar to those of Krebs and Johnson. The enzymatic conversion of 2,4-diketoundecylate to octanoate and pyruvate has also been demonstrated. These data are compatible with the following general equation in which hydrolysis of an acylpyruvic acid yields 1 mole each of pyruvic acid and the corresponding fatty acid.



The term "acylpyruvase" is proposed for the enzyme or enzymes which catalyze this reaction. If the rates of hydrolysis of the aliphatic diketo acids (Table IV) are plotted as a function of carbon chain length, two maxima are obtained which correspond to substrates with 6 and 10 carbon atoms in the chain. The introduction of methyl groups in the 5 position of the diketohexanoic acid molecule resulted in decreases in rate of 37 and 74 per cent for the 5-methyl and 5,5-dimethyl derivatives, respectively. Similarly, 2,4-diketo-6-methylheptanoic acid was hydrolyzed more slowly than the normal 7-carbon diketo acid. The optimal rate for hydrolysis of the 5-, 6-, and 7-carbon normal diketo acids occurs at the pH range 7.2 to 7.9, while that for the normal diketo acids containing between 8 and 11 carbon atoms is pH 8.0 to 8.9. Under the conditions employed 2,4-diketohexanoate and 2,4-diketocaproate are hydrolyzed more rapidly than the other diketo acids studied and in addition these substrates have significantly different pH optima. These findings suggest the possibility of the existence of more than one acylpyruvase. However, similar activity ratios were obtained with the partially purified enzyme preparation. It is possible that other factors may be responsible for these findings and further study of the possible multiplicity of acylpyruvase is necessary.

The rôle, if any, of acylpyruvase in intermediary metabolism remains to be elucidated. Apparently 2,4-diketo acids have not yet been isolated from biological material, although a closely related compound, α -keto- γ -hydroxyvaleric acid, was isolated from the products of pyruvic acid fermentation by *Staphylococcus albus* by Fosdick and Rapp (27). The possibility of a reversal, under certain circumstances, of the equation given above with the formation of 2,4-diketo acids from pyruvic acid and fatty acid, as originally suggested by Krebs and Johnson for pyruvic and acetic acids, must be considered. This is of special interest in view of the acceleration by pyruvate of the incorporation of acetate carbon into liver fatty acids (28). The occurrence of α, γ oxidation of fatty acids has never been demonstrated, although Jowett and Quastel considered this mechanism for the oxidation of odd carbon fatty acids (29). The possibility of α oxidation has also been considered by Witzemann (30). Breusch and Ulusoy have reported that 3,5-diketohexanoic acid is converted to acetoacetate when incubated with cat liver suspensions (23). Kidney suspensions were inactive, as were those of other organs. These authors found that 1.3 moles of acetoacetate were formed from each mole of substrate, this result being considered in harmony with the β oxidation-condensation theory. Breusch and Ulusoy conclude that α, γ oxidation of fatty acids is unlikely, since

when 2,4-diketohehexanoic acid was incubated with cat liver suspensions no trace of acetoacetate was formed. However, the present data indicate that 2,4-diketohehexanoic acid is hydrolyzed to yield pyruvic acid under conditions similar to those of these workers. The reluctance of previous workers to pursue the possibility of α, γ oxidation of fatty acids is understandable in the light of impressive evidence supporting the β oxidation theory. However, evidence excluding the occurrence of α, γ oxidation with subsequent pyruvate formation as a possible alternative pathway of fatty acid oxidation has not yet been reported, and, in view of the present findings, further work along these lines seems desirable.

The authors wish to thank Mr. Charles Kinser for performing the elemental analyses.

SUMMARY

It has been found that extracts of liver and kidney catalyze the hydrolysis of 2,4-diketovaleric acid, yielding nearly equivalent amounts of pyruvic and acetic acids. This reaction occurs aerobically and anaerobically, and without detectable acetoacetate formation. The effects of substrate and liver extract concentration on the rate of hydrolysis have been studied. Formation of pyruvic acid from 2,4-diketovaleric acid has also been demonstrated in liver slices. A series of eleven other 2,4-diketo acids is hydrolyzed in a similar manner to yield pyruvic acid. The hydrolysis of 2,4-diketoundecylic acid resulted in the appearance of equimolar amounts of pyruvic and octanoic acids. Of the diketo acids studied, 2,4-diketohehexanoic and 2,4-diketocapric acids are hydrolyzed most rapidly. The pH optima for hydrolysis of the normal aliphatic 2,4-diketo acids are in the range 7.2 to 7.9 for the 5-, 6-, and 7-carbon acids, and 8.0 to 8.9 for the acids containing between 8 and 11 carbon atoms. The term "acylpyruvase" is proposed for the enzyme or enzymes which catalyze 2,4-diketo acid hydrolysis. Acylpyruvase activity is present in the liver and kidney of the rat, rabbit, cat, and mouse. All other tissues studied, including primary rat hepatoma, possess little enzymatic activity. A partially purified enzyme preparation has been obtained from rat liver.

Methods of preparation of the disodium salts of 2,4-diketo acids are given. The ultraviolet absorption spectra for the ethyl esters of aliphatic and aromatic 2,4-diketo acids and the relationship between enolization and ultraviolet absorption are described. A spectrophotometric method for the determination of acylpyruvase activity is presented.

Implications of these findings in terms of intermediary metabolism are discussed.

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CYTOCHEMICAL STUDIES ON THE CHICKEN ERYTHROCYTE

I. AMINO ACID CONTENT AND DISTRIBUTION*

By R. M. MELAMPY

(From the Department of Physiology, University of Illinois, Urbana)

(Received for publication, April 29, 1948)

Chicken erythrocytes possess many characteristics which make them a desirable material for experimental studies in cytochemistry, particularly those dealing with the chemical composition of various structural components within the cell. Avian erythrocytes have been used as a convenient source of material for the isolation of nuclei. Investigations along such lines were reported as early as 1869 by Plosz (1) and Ackermann (2), in 1904, prepared nuclei from chicken erythrocytes by laking cells in distilled water and precipitating the nuclei in 3.6 per cent sodium chloride. Warburg (3) used freezing and thawing as a technique for isolating nuclei. More recently, however, Laskowski (4) recommended lysolecithin as a hemolyzing agent and Dounce and Lan (5) suggested saponin for this purpose. Isolated chicken erythrocyte nuclei have been used as a source of material for recent biochemical studies on the composition of chromosomes (6), electron microscope studies of isolated chromosomes (7), and studies on the influence of irradiation on the respiration of nuclei (8).

This report presents data on the distribution of certain amino acids in the proteins of the nucleus and cytoplasm of the chicken erythrocyte. Data are also given on the dry weight and nitrogen content of whole cells and isolated nuclei.

Materials and Methods

Preparation of Erythrocytes—Five samples of dried erythrocytes, each weighing about 2.5 gm., were prepared and used for the determination of amino acids and total nitrogen. The method for collecting the blood as well as the procedure for washing and drying the cells is described in an earlier report (9).

Preparation of Nuclei—Five lots of erythrocytes were prepared and after the last washing were resuspended in buffered saline containing 0.3 per cent

* This work was supported by funds from the Research Board of the University of Illinois. Acknowledgment is made of the help of Professor H. H. Mitchell. Sodium pteroylglutamate used in the assay media was supplied by Dr. T. H. Jukes, Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

Merck's purified saponin as used by Dounce and Lan (5). The erythrocytes were allowed to stand with occasional stirring for 20 to 30 minutes until laked. The isolated nuclei were then washed 10 times with physiological saline adjusted to pH 7.0 with M/15 phosphate buffer. Nuclei prepared in this way were slightly yellow in color, and in microscopic appearance were similar to those prepared by Laskowski and Ryerson (10). A hematocytometer was used to make the nuclear count. It must be emphasized that about 20 per cent of the nuclei in our preparations possessed tenuous stromata; this observation confirms the earlier findings of Dounce and Lan (5) and Laskowski and Ryerson (10). These nuclear preparations were dried at 105° and stored for chemical analyses and microbiological assays.

Preparation of Hydrolysates—All materials were dried to constant weight before samples were taken for hydrolysis. 1 gm. samples of dried erythrocytes and nuclei were transferred to vials prepared by drawing out 150 × 22 mm. Pyrex test-tubes. To each vial 10 ml. of 10 per cent (by volume) hydrochloric acid were added. The vials were sealed and autoclaved for 10 hours at 15 pounds pressure. After being cooled, the ampuls were broken and the hydrolysate was washed into a beaker with a small amount of water. The hydrolysate was neutralized with 5 N sodium hydroxide, the pH adjusted to 6.8, and the solution was filtered and diluted to a final volume of 50 ml. with water. The hydrolysates were stored under toluene in a refrigerator and aliquots were diluted with distilled water before being assayed.

Assay Procedures—Microbiological assays were used to determine the amounts of the various amino acids present in the hydrolysates. The histidine, arginine, and lysine analyses reported here were made by the Shankman Laboratories, Los Angeles, California; the assay procedure for arginine was similar to that of McMahan and Snell (11) except that the serine content of the basal medium was increased 50 per cent and hydroxyproline omitted. *Lactobacillus casei* was the test organism. Lysine and histidine were assayed with the medium described by Dunn *et al.* (12), with *Leuconostoc mesenteroides* P-60.

The method for the determination of amino acids as developed by Stokes, Gunness, Dwyer, and Caswell (13), with *Streptococcus faecalis*, was employed to determine leucine and isoleucine in the erythrocytes and tryptophan in both the whole cells and the isolated nuclei. The valine and threonine contents of the erythrocytes were determined with *Lactobacillus arabinosus* 17-5 according to Hier, Graham, Freides, and Klein (14). The medium of Schweigert, McIntire, Elvehjem, and Strong (15) with *Lactobacillus arabinosus* 17-5 as the assay organism was used for methionine and phenylalanine and, in addition to these, for leucine, isoleucine, valine, and

threonine in the hydrolysates of the nuclei. Tyrosine was determined according to the method of Gunness, Dwyer, and Stokes (16), with *Lactobacillus delbrueckii* LD5.

TABLE I

Amino Acids and Total Nitrogen of Dried Chicken Erythrocytes and Nuclei

The results are expressed in mg. per gm.

Amino acids	Erythrocytes		Nuclei	
	Mean	Standard deviation	Mean	Standard deviation
Histidine.....	51	0.6	9	0.4
Arginine.....	47	0.9	52	2.3
Lysine.....	75	1.2	44	1.6
Leucine.....	92	6.0	38	0.3
Isoleucine.....	32	0.7	26	0.8
Valine.....	71	2.9	25	0.5
Methionine.....	13	0.9	9	0.5
Threonine.....	49	1.6	19	1.1
Tryptophan.....	13	0.3	2	0.0
Phenylalanine.....	43	1.4	12	0.2
Tyrosine.....	26	2.3	17	0.8
Total N, micro-Kjeldahl..	15.7	3.0	11.4	0.9
“ “ Dumas.....	15.9	0.9	11.3	0.9

TABLE II

Nuclear and Cytoplasmic Distribution of Amino Acids in Chicken Erythrocyte

	$\gamma \times 10^{-3}$ per dry erythrocyte			Cytoplasmic Nuclear ratio
	Whole cell	Nucleus	Cytoplasm	
Histidine.....	2.3	0.10	2.20	22.0
Arginine.....	2.1	0.60	1.50	2.5
Lysine.....	3.3	0.50	2.80	5.6
Leucine.....	4.1	0.40	3.70	9.3
Isoleucine.....	1.4	0.30	1.10	3.7
Valine.....	3.2	0.30	2.90	9.7
Methionine.....	0.6	0.10	0.50	5.0
Threonine.....	2.2	0.20	2.00	10.0
Tryptophan.....	0.6	0.03	0.57	19.0
Phenylalanine.....	1.9	0.10	1.80	18.0
Tyrosine.....	1.2	0.20	1.00	5.0

Results

The data obtained from the analyses of five samples of whole erythrocytes and isolated nuclei are presented in Table I. The total nitrogen values

were obtained by the micro-Kjeldahl and Dumas methods on moisture-free samples. It is to be noted that the values are practically the same with both procedures. Total nitrogen was also determined on a moisture-free, lipide-free basis, and in the case of the erythrocytes it was 15.8 per cent and in the nuclei 12.5 per cent. The nuclear material constituted 23.7 per cent of the dry weight of the erythrocytes (10.5×10^{-6} γ per cell). By using the average dry weight per single cell of 44.3×10^{-6} γ , it was possible to calculate the amounts of the various amino acids contained in the proteins of the cytoplasm which are removable by hemolysis with saponin and the amounts of those present in the nuclear material. The nuclear and cytoplasmic distribution of the amino acids determined in this work is presented in Table II.

DISCUSSION

A comparison of the amino acids contained in the whole erythrocytes with those in isolated nuclei, as given in Table I, shows that cells are higher than nuclei in histidine, lysine, leucine, isoleucine, valine, methionine, threonine, tryptophan, phenylalanine, and tyrosine. On the other hand nuclear material is somewhat higher in arginine. It is also of interest that the cells contain approximately 6 times more histidine and tryptophan than does nuclear material.

The data presented in Table II are more significant than those in Table I in evaluating the chemical basis of the organization of this cell, particularly the cytoplasmic-nuclear ratio of the various amino acids. However, the functional significance cannot be determined at this time. Mirsky and Ris (17) in their work on the composition of chromosomes have found a low tryptophan content in the histones of fowl erythrocytes. The results presented here confirm this finding, as the tryptophan content of a single nucleus is 0.03×10^{-6} γ , which represents the smallest quantity of any of the amino acids determined. Arginine is the most abundant amino acid in these nuclear preparations (0.60×10^{-6} γ). Leucine is present in relatively large amounts (3.70×10^{-6} γ) in the cytoplasm, whereas the concentration of methionine (0.50×10^{-6} γ) is one of the lowest according to the data in Table II. It should be mentioned that hemoglobin contributes a major portion of the amino acids found in the cytoplasm of the chicken erythrocyte. Furthermore, in evaluating the analytical data presented in Tables I and II it is necessary to stress that the residual stromata retained by the nuclei have contributed to the composition of the material analyzed.

SUMMARY

The distribution of several amino acids in the nuclear and cytoplasmic proteins of the chicken erythrocyte has been determined by use of microbiological assays.

The total nitrogen content of chicken erythrocytes on a moisture-free, lipid-free basis was 15.8 per cent nitrogen and of the isolated nuclei 12.5 per cent. The average dry weight of a single erythrocyte was $44.3 \times 10^{-6} \gamma$ and of a nucleus $10.5 \times 10^{-6} \gamma$.

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A SPECIFIC COLOR REACTION OF METHYLPENTOSEs AND A SPECTROPHOTOMETRIC MICROMETHOD FOR THEIR DETERMINATION*

ZACHARIAS DISCHE AND LANDRUM B. SHETTLES

(From the Department of Biochemistry and the Department of Obstetrics, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, April 13, 1948)

Methylpentoses have been known for a long time as constituents of polysaccharides of plant and bacterial origin. Only recently fucose was found in the animal body as a constituent of blood group substances, combined with other sugars. These polysaccharides seem to be widely distributed in animal tissues and probably are of biological importance. No method has so far been available for determining small amounts of methylpentoses in the presence of other sugars. Dische (1) recently described a group of color reactions of carbohydrates with SH compounds in H_2SO_4 . These color reactions are characteristic for various classes of carbohydrates (desoxypentoses, pentoses, methylpentoses, hexoses, hexuronic acids) and for individual hexoses and hexuronic acids. This report deals with one of these reactions which was found usable for the detection and determination of 2 to 10 γ of methylpentose in the presence of a considerable excess of other sugars.

EXPERIMENTAL

Simple Qualitative Test

Procedure—To 1 cc. of a solution containing 50 γ or more of a methylpentose in a 16 $\times$ 150 mm. test-tube are added with cooling in ice 4.5 cc. of a mixture of 1 volume of H_2O and 6 volumes of H_2SO_4 , c.p. The mixture is then warmed to 20–22° for a few minutes, held for either 3 or 10 minutes in an actively boiling bath, and finally cooled in tap water. To the cold solution 0.1 cc. of 3 per cent aqueous cysteine hydrochloride is added with shaking. A greenish yellow color appears and remains practically unchanged for 24 hours.

Specificity of Reaction—As the color developed in the cysteine reaction depends upon the time of heating, we have distinguished two types of the reaction, designating them by CyR3 and CyR10, respectively. The green-yellow color is characteristic for methylpentoses in only CyR10. Here pentoses, hexoses, and hexuronic acids give a pink color. In CyR3 hexoses show also a yellow color. This, however, decreases rapidly in intensity (in contrast to the reaction obtained with methylpentoses) and, except in

* This work was supported by a grant of the Donner Foundation, Inc., Cancer Research Division.

the case of mannose, changes into another color: green for glucose and fructose, blue for galactose and sorbose. Apart from this instability the yellow reaction product of hexoses differs from that produced by methylpentoses in its absorption spectrum.

Spectrophotometric Test for Methylpentoses

Absorption Spectra of CyR3 and CyR10 of Various Sugars—Whereas CyR10 appears more specific than CyR3, the latter has the advantage of making it possible to detect and to determine hexoses as well as methylpentoses at the same time in one sample. For this reason the absorption spectra for both types of CyR are given in Figs. 1 and 2. For spectrophotometric measurements a much lower concentration of methylpentoses can be used than for the qualitative test. In our experiments they ranged between 2 and 10 γ per cc. The absorption spectra of methylpentoses are almost identical in both types of CyR. They show a sharp maximum at 4000 A and the range of the absorption is between 3600 and 4300 A. In the 10 minute reactions the curves of pentoses and hexuronic acids are practically horizontal in this range. Desoxyribonucleic acid shows a weak maximum at 3800 A and glucose a weak, barely perceptible maximum at 4150 A, with very high values of transmission in the whole range. In the 3 minute reaction, however, all hexoses show a strong absorption between 3600 and 4300 A, with an almost symmetrical peak at 4150 A, while the other sugars behave as in CyR10.

Absorption Increment between Two Wave-Lengths As Test for Methylpentose—These great differences in the absorption spectra of CyR of various classes of sugars enable us to detect 2 to 10 γ of methylpentose in the presence of an excess of other sugars in the following way. As can be seen from Figs. 1 and 2 and Tables I and II, the CyR of methylpentoses shows practically no absorption at 4300 A. On the other hand, hexoses show in CyR3 a considerable absorption at this wave-length, owing to the difference in the position of the maximum of the absorption curve, and a small but definite absorption in CyR10. Because of the symmetric shape of the absorption curve of hexoses with regard to the peak at 4150 A, the absorption at 4300 A is equal to that at 3960 A.¹ At this latter wave-

¹ In some experiments carried out recently, the wave-length at which the absorption of CyR3 of hexoses was equal to E_{3960} was not 4300A but 4270A. These small variations were probably due to variations of the time necessary to bring the reaction mixture to 100° after immersion in boiling water. For the detection and determination of methylpentoses in solutions containing hexoses it is, therefore, necessary to run in every determination a hexose standard and find the exact wave-length at which E CyR3 of hexoses equals E_{3960} . Because of the identical shape of absorption curves of CyR3 of all four natural hexoses, any one of them can be used as standard for this purpose, no matter which one is present in solution.

length the methylpentoses show almost maximum absorption. The difference between the extinction coefficients at 3960 Å and at 4300 Å,

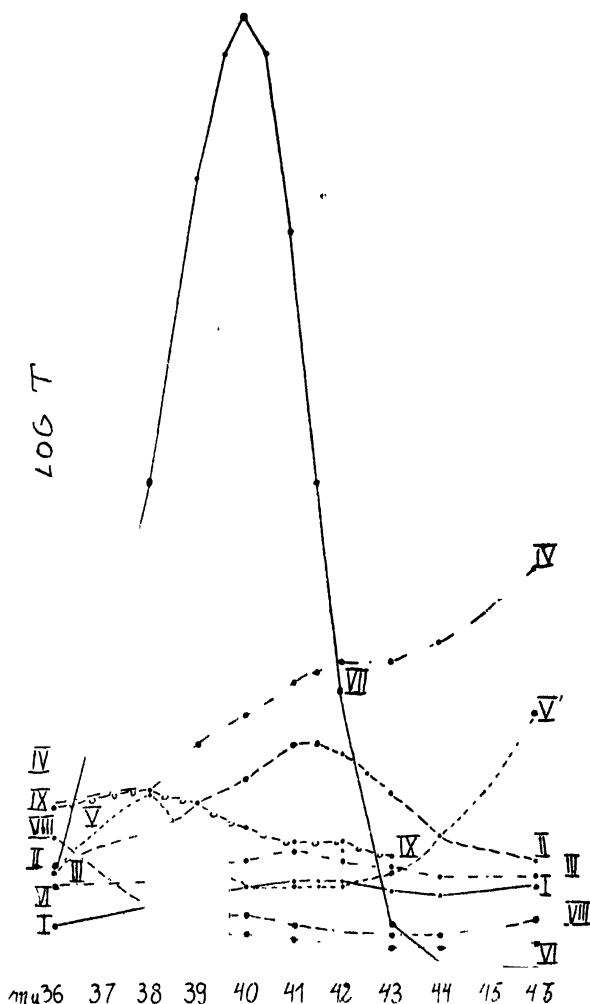


FIG. 1. Absorption spectra of CyR10 of various sugars determined with a Beckman spectrophotometer. Curve I, arabinose 5 mg. per cent; Curve II, glucose 5 mg. per cent; Curve III, mannose 5 mg. per cent; Curve IV, fructose 5 mg. per cent; Curve V, desoxyribonucleic acid 25 mg. per cent; Curve VI, glucuronic acid 10 mg. per cent; Curve VII, fucose 1 mg. per cent; Curve VIII, galacturonic acid 10 mg. per cent; Curve IX, galactose 5 mg. per cent.

$E_{3960} - E_{4300}$, designated $\Delta E_{3960-4300}$, is therefore in both types of CyR highly positive for methylpentoses. 10 γ of fucose in 1 cc. of the unknown solu-

tion give $\Delta E_{3960-4300}$ of 220 to 230. $\Delta E_{3960-4300}$ is practically zero in CyR10 for desoxyribose, hexoses, pentoses, and hexuronic acids (Table I). In CyR3 it is zero for hexoses and for desoxyribose and slightly negative for hexuronic acids; pentoses give in CyR3 a slightly positive value, which, however, corresponds to no more than 1 per cent of the value for an

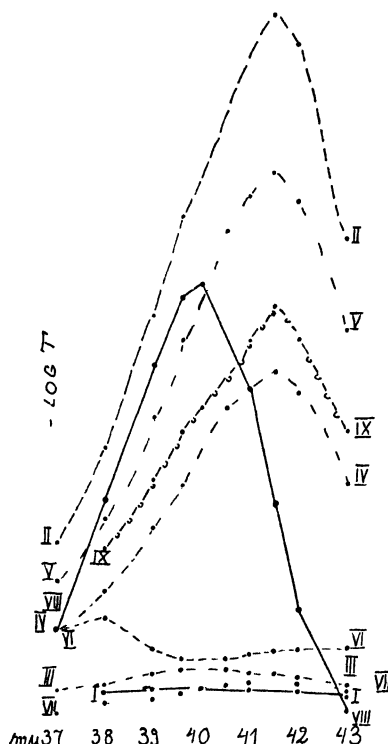


FIG. 2. Absorption spectra of CyR3 of various sugars determined with a Beckman spectrophotometer. Curve I, arabinose 5 mg. per cent; Curve II, glucose 5 mg. per cent; Curve III, yeast adenylic acid 10 mg. per cent; Curve IV, mannose 5 mg. per cent; Curve V, fructose 5 mg. per cent; Curve VI, desoxyribonucleic acid 50 mg. per cent; Curve VII, glucuronic acid 10 mg. per cent; Curve VIII, fucose 1 mg. per cent; Curve IX, galactose 5 mg. per cent.

equivalent amount of methylpentose. As pentoses show a pink CyR with an absorption maximum at 5400 Å, any significantly positive $\Delta E_{3960-4300}$ due to pentoses will be accompanied by a distinctly pink color of the reaction mixture. Any positive value of $\Delta E_{3960-4300}$ which cannot be accounted for by a corresponding amount of pentoses, indicates, therefore, in both types of CyR the presence of methylpentoses in solution. As can be seen from Tables I and II, $\Delta E_{3960-4300}$ of a methylpentose is not affected at all by a 4-fold excess of a hexose or an 8-fold excess of a pentose.

Quantitative Determination of Methylpentoses in Solutions

$\Delta E_{2980-4300}$ As Quantitative Measure for Methylpentoses—It can be seen from Tables I and II that $\Delta E_{2980-4300}$ of a methylpentose is strictly proportional to the concentration of the sugar. As stated above, it is not

TABLE I
Extinction Coefficients at 4300 and 2980 Å and Their Differences for CyR10
of Various Sugars

Readings 1 hour, 30 minutes to 2 hours, 30 minutes after the addition of cysteine; Beckman spectrophotometer.

Experiment No.	Substance	Concentration	CyR10		
			E_{4300}	E_{2980}	$E_{2980} - E_{4300}$
1	Glucose	5	0.039	0.039	0
	Fructose	5	0.066	0.053	-0.013*
	Mannose	5	0.024	0.024	0
	Galactose	5	0.026	0.033	+0.007
	Arabinose	5	0.020	0.019	-0.001
	Desoxyribose nucleic acid	25	0.025	0.021	-0.004
	Glucuronic acid	10	0.007	0.010	+0.003
	Galacturonic acid	10	0.010	0.014	+0.004
	Fucose	1	0.012	0.192	+0.180
2	Fucose	1	0.011	0.195	+0.184
	"	0.5	0.005	0.097	+0.092
	"	2	0.021	0.363	+0.342
	Galactose	2.5	0.010	0.012	+0.002
	"	2.5	0.016	0.110	+0.094
	+ fucose	0.5			
	Arabinose	5	0.015	0.015	0
	"	5	0.025	0.115	+0.090
	+ fucose	0.5			
	+ "	0.5			
	+ serum albumin	50	0.004	0.099	+0.095

* Fructose reacts under conditions of CyR10 with sulfuric acid alone, giving a pink color. The absorption of this reaction measured against a blank without cysteine was subtracted from the absorption of the sample containing cysteine. Other sugars do not react significantly with H_2SO_4 alone. The absorption spectrum of fructose in Fig. 1 is not corrected in this way.

influenced by a considerable excess of hexoses or pentoses. By comparing $\Delta E_{2980-4300}$ of an unknown solution with that of a standard solution of fucose or rhamnose, it should be possible to determine methylpentoses in solutions like hydrolysates of polysaccharides containing other sugars.

TABLE II

Extinction Coefficients at 4300 and 3960 Å and Their Differences for CyR3 of Various Sugars

Readings 1 hour, 30 minutes to 2 hours, 30 minutes after the addition of cysteine; Beckman spectrophotometer.

Experi- ment No.	Substance	Concen- tration <i>mg. per cent</i>	E_{4300}	E_{3960}	$E_{3960} - E_{4300}$
1	Fucose	1	0.007	0.222	+0.215
	"	0.5	0.003	0.113	+0.110
	Galactose	2.5	0.074	0.074	0
	"	2.5	0.077	0.292	+0.215
	+ fucose	1			
	Glucose	2.5	0.117	0.118	+0.001
2	Glycogen	5	0.291	0.293	+0.002
	Fucose	1	0.056	0.299	+0.243
	"	1	0.052	0.296	+0.244
	+ ribonucleic acid	10			
	Hyaluronic acid	25	0.026	0.031	+0.005
	Chondroitinsulfuric acid	30	0.001	0.013	+0.012
3	Desoxyribonucleic acid	20	0.046	0.045	-0.001
	Glucose	25	0.145	0.146	+0.001
	Ribonucleic acid	22.5	0.027	0.039	+0.012
4	Fucose	1	0.008	0.231	+0.223
	"	1			
	+ galactose	4	0.145	0.362	+0.217
	+ acetylglucosamine	5			
	Rhamnose	1	0.007	0.212	+0.205
5	Glucose	5	0.254	0.265	+0.011
	Yeast adenylic acid	10	0.019	0.028	+0.009
	Mannose	5	0.127	0.126	-0.001
	Fructose	5	0.205	0.201	-0.004
	Desoxyribose nucleic acid	50	0.040	0.035	-0.005
	Muscle adenylic acid	5	0.010	0.015	+0.005
	Glucuronic acid	10	0.016	0.017	+0.001
	Galacturonic acid	20	0.044	0.052	+0.008
6	Fucose	1	0.008	0.233	+0.225
	"	1			
	+ arabinose	8	0.028	0.260	+0.232
	Fucose	0.5	0.003	0.114	+0.111
	"	0.25	0.002	0.058	+0.056
	"	0.5			
	+ serum albumin	100	0.032	0.140	+0.108

TABLE II—*Concluded*

Experiment No.	Substance	Concentration	E_{2900}	E_{3300}	$E_{3300} - E_{2900}$
		mg. per cent			
7	Blood group substance A	10	0.098	0.255	+0.157
	" " " "	20	0.211	0.530	+0.317
	Fucose	1			
	+ galactose	2.5	0.085	0.297	+0.212
	+ acetylglucosamine	5			
8	Hydrolysate of blood group substance A	10	0.095	0.220	+0.125
	Fucose	1			
	+ galactose	4	0.135	0.341	+0.206
	Mixture of blood group substances	10	0.067	0.232	+0.165

It is necessary to run a standard every time with the unknown and make the readings at least 1 hour after addition of cysteine.¹

Determination in Polysaccharides—To test the possibility of determining methylpentoses in non-hydrolyzed polysaccharides $\Delta E_{2900-3300}$ was determined with CyR3 in solutions of two preparations of blood group substances from hog stomach mucosa.² Preparation I was obtained from the mucosa of several stomachs and represented a mixture of blood group substances A and O. Preparation II was prepared from one single stomach and purified so that it contained only substance A. The values obtained for fucose were 6.7 per cent for Preparation I and 7.75 per cent for Preparation II. A hydrolysate of Preparation II gave 5.9 per cent of fucose (Table II). As a certain amount of fucose is undoubtedly destroyed during hydrolysis, it is impossible to estimate the accuracy of the values obtained with unhydrolyzed preparations. However, since these values are proportional to the amount of polysaccharide (see Table II), it is clear that the method can be used safely for comparative determinations in various preparations of a certain type of polysaccharide.

Determination in Substrates of Biological Origin—When CyR is used for determinations of methylpentoses in materials of biological origin such as tissue extracts, it must be kept in mind that many organic substances give colored reaction products when heated with sulfuric acid. In these cases therefore it is necessary also to prepare samples of the unknown solution of the standard and a water blank to which no cysteine is added after heating with H_2SO_4 . The absorption due to CyR is obtained by subtracting the absorption of a sample without cysteine from that with cysteine. The

¹ We are greatly indebted to Dr. Karl Meyer and Dr. Elvin A. Kabat for the preparation of blood group substances.

absorption of the sample of the unknown without cysteine is measured against the water blank without cysteine. Certain body fluids like various kinds of mucus contain in general considerable amounts of proteins. In these substances it is not always possible to separate the polysaccharides quantitatively from proteins by the usual deproteinization procedures. The influence of 0.05 to 0.10 per cent solutions of serum albumin on $\Delta E_{3960-4300}$ was studied. As proteins give brown-colored products when heated with H_2SO_4 alone, the absorption due to this latter reaction was measured and subtracted from the absorption due to CyR10 and CyR3. As can be seen in Table I, 0.05 per cent of serum albumin did not affect the $\Delta E_{3960-4300}$ of 0.031 per cent fucose. 0.1 per cent decreased it by only 3 per cent. Furthermore, in general when working with body fluids or tissues it is necessary to find out whether methylpentose added to the substrate can be recovered. If the substrate decreases or increases the $\Delta E_{3960-4300}$ of added methylpentose, it becomes necessary to compare $\Delta E_{3960-4300}$ of the unknown solution not with that of a pure solution of the methylpentose, but with $\Delta E_{3960-4300}$ given by a certain amount of methylpentose added to the unknown solution (internal standard).

DISCUSSION

The investigation of the specificity of our reaction has so far been confined to those sugars which are commonly found in biological materials. It seems possible that other substances like branched or anhydro sugars, found less commonly or not at all in nature, may react with cysteine like methylpentoses. At present, therefore, the only conclusions that can safely be drawn relate to the absence of methylpentoses in an unknown material.

However, the certainty of any identification of a sugar by color reactions involving decomposition of the sugar by strong acid can be enhanced considerably if the quantitative determination of the respective sugar by two completely different color reactions yields identical results. This becomes clear when certain general features of the mechanism of this type of color reaction are considered. Every one of these reactions consists of two more or less independent steps: (1) the breakdown of the sugar and (2) the combination of products of this breakdown with certain organic "developers" (carbazole, indole, diphenylamine, SH compounds) to yield colored reaction products. The great differences in the intensity and quality of these color reactions between different classes of sugars and individual sugars indicate that the breakdown of the sugar by the action of strong acid leads to a great variety of reaction products. The nature and quality of these products depend not only on the nature of the sugar but on the conditions of the reactions, like strength of the acid, temperature, and time of heating (2). These conclusions are borne out by the work of Stacey (3) and as-

sociates on the chemistry of the diphenylamine reaction of desoxypentose. Further supporting evidence will be given later in a report on the general cysteine reaction of sugars.

Under these circumstances it is improbable that two classes of sugars should both give respectively identical absorption in the same range of their spectra in two different reactions. It, therefore, appears important in any preliminary identification to estimate the amount of the individual sugars by two color reactions, differing in temperature, time of heating, and concentration of the acid.

The question of the quantitative accuracy of this reaction when applied to non-hydrolyzed polysaccharides cannot be settled by a comparison with results obtained on hydrolysates, because of the possibility of decomposition of sugar during hydrolysis. The extent of this may depend on specific linkages in the polysaccharide and cannot be calculated safely by controls on pure sugar solutions. If, however, the determination of the respective sugar in the non-hydrolyzed polysaccharide by two different methods gives identical results, it is fairly safe to assume that these values are correct. Under any circumstances, however, the procedure can be used for comparative determination of methylpentoses in various preparations and tissue extracts.

In the animal body a methylpentose, fucose, has been found as a constituent of the so called blood group substances. It is combined here with galactose. The detection of a methylpentose in tissues or body fluids will, therefore, suggest the presence of blood group substances. This inference would be strengthened by finding an appropriate amount of galactose in the same material. It is possible to determine hexoses by their cysteine reaction in the same sample in which the determination of methylpentoses is carried out. A procedure for this purpose will be reported in a subsequent paper.

SUMMARY

1. A new characteristic color reaction of methylpentoses is described.
2. A spectrophotometric micromethod for the detection and determination of methylpentoses in the presence of an excess of other sugars is elaborated.

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NITROSOTOCOPHEROLS; THEIR USE IN THE CHEMICAL ASSAY OF THE INDIVIDUAL TOCOPHEROLS IN A MIXTURE OF THE α , β , γ , AND δ FORMS*

BY MARY LOUISE QUAIFE

(From the Research Laboratories of Distillation Products, Inc., Rochester, New York)

(Received for publication, April 29, 1948)

Four naturally occurring members of the vitamin E group greatly different in biological activity are now known, and it is desirable to have analytical methods by which to differentiate them. In this paper is described a procedure by which three of them can be specifically determined. It involves the formation, chromatographic separation, and photometric measurement of the nitroso derivatives of β -, γ -, and δ -tocopherols. α -tocopherol can be estimated by difference between total tocopherols and the sum of β -, γ -, and δ -tocopherols.

We have found that alcoholic solutions of β -, γ -, and δ -tocopherols, but not α -tocopherol, form yellow nitroso derivatives on treatment with nitrous acid.¹ The rate of color development differs markedly for the three "non- α -tocopherols" as shown in Fig. 1. On treatment with alkali the nitrosotocopherols turn red. Measurement of color at this stage is not suitable as a method, since the aqueous-alcohol solution would be turbid due to fat which accompanies vitamin E in biological materials. However, on dilution with water and extraction into petroleum ether (Skellysolve H), the nitrosotocopherols give clear, stable yellow solutions suitable for assay purposes.

The spectra of the nitrosotocopherols are shown in Fig. 2. They are very similar to those of some *o*-nitrosophenols given by Cronheim (2). Moreover the positions of absorption maxima and minima appear to obey his rules, *e.g.*, (Rule 1) the wave-length of minimum absorption increases with the number of substituents in the benzene ring, and (Rule 2) the wave-length of maximum absorption depends upon the position relative to the hydroxyl group of the nearest substituent (except the nitroso group). Thus, as an illustration of Rule 1, the nitroso derivative of δ -tocopherol has an absorption minimum at 340 $m\mu$, while nitroso- β - and nitroso- γ -tocopherols, with one more methyl group each, have absorption minima at

* Communication No. 132 from the Research Laboratories of Distillation Products, Inc.

¹ That nitrous acid gives a color with β - and γ -tocopherols, but not α -tocopherol, dissolved in alcoholic solution was noted by Scudi and Buhs (1), who attributed the color to formation of tocopherylquinone.

about 355 $m\mu$. To illustrate Rule 2 Cronheim gives the wave-lengths of the absorption maxima of nitrosophenols classified according to the position of the nearest substituent, other than nitroso, relative to the hydroxyl group. These are para, 390 to 395 $m\mu$; meta, 400 to 405 $m\mu$; ortho, 410 to 420 $m\mu$. The nitrosotocopherols correspond exactly to this classification. Absorption maxima occur at 410, 415, and 405 $m\mu$, respectively, for nitroso β -, γ -, and δ -tocopherols, and the former two are ortho-substituted while the latter is meta.

Red forms of the nitrosophenols can be produced in petroleum ether by

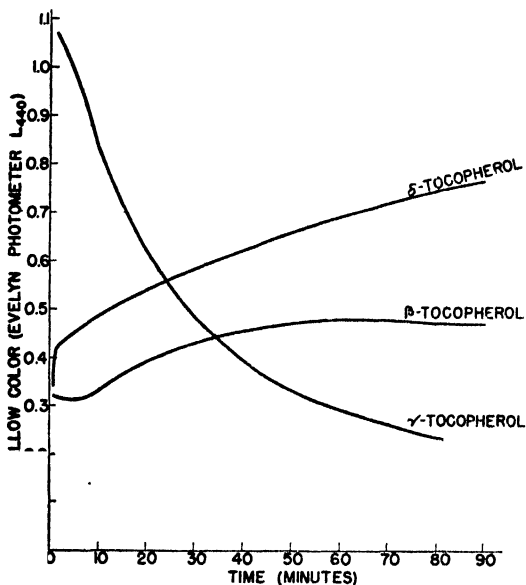


FIG. 1. Nitrosation of tocopherols in ethanol solution; color change with time. Each solution contained 1.5 mg. of tocopherol dissolved in 5 ml. of ethanol, 0.2 ml. of glacial acetic acid, and 3 ml. of 2 per cent sodium nitrite (aqueous)

shaking with a small amount of base such as trimethylbenzylammonium hydroxide. The colors are not stable.

Unlike other *o*-nitrosophenols, the nitrosotocopherols do not appear to give metallic complexes. Petroleum ether solutions of the nitrosotocopherols, when shaken with aqueous solutions of Cu^{++} or Co^{++} , adjusted to pH near neutrality, gave no color change or precipitate. Under the same conditions, β -nitroso- α -naphthol gave voluminous precipitates.

The procedure which has been developed for the assay of the individual or of combined non- α -tocopherols is as follows.

Reagents—

Glacial acetic acid.

Sodium nitrite (2 gm. per 100 ml. of distilled water). Keep in the ice box. Let warm to room temperature before using. Make fresh every few days.

Potassium hydroxide (20 gm. per 100 ml. of distilled water).

Anhydrous sodium sulfate.

Skellysolve H.

Method—All operations should be performed under subdued artificial light.

Place exactly 5 ml. of an absolute ethanol solution of the oil containing at least 0.25 mg. and not more than 1 mg. of non- α -tocopherols in a 50 ml. glass-stoppered graduated cylinder. Add exactly 0.2 ml. of glacial acetic

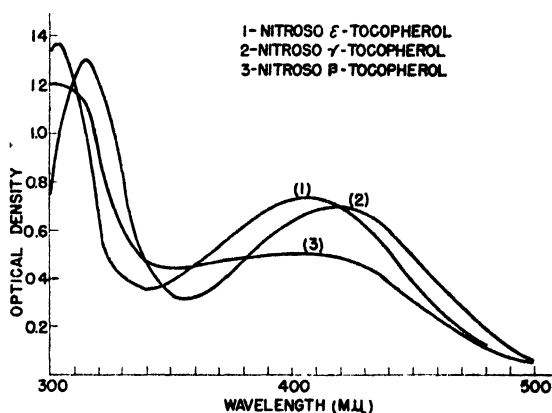


FIG. 2. Absorption spectra of the nitrosotocopherols in Skellysolve H. These were determined with the Beckman spectrophotometer on regular assay solutions with a similar blank. Concentration in each solution was approximately 1.5 mg. of tocopherol per 12 ml. of solution.

acid. Swirl to mix. Add exactly 3 ml. of sodium nitrite reagent from a fast delivery pipette. Swirl vigorously for 5 seconds to mix. Let stand exactly 60 seconds. Add 2 ml. of potassium hydroxide reagent and mix. Add roughly 10 ml. of distilled water, a pinch of anhydrous sodium sulfate, and exactly 12 ml. of Skellysolve H. Stopper, shake vigorously for 30 seconds, and let settle. Transfer at least 10 ml. of the upper layer to an Evelyn photometer tube and read with Filter 400 against a blank which has been prepared in exactly the same fashion. Or determine the density at 410 $m\mu$ in the Beckman spectrophotometer, using a similar blank.

Calculation—For Evelyn photometer readings, $C = L_{400} \times K$, where C = mg. of non- α -tocopherols in the original 5 ml. of alcoholic solution and $L_{400} = 2 - \log G_{400}$. For Beckman spectrophotometer readings use a calibration curve. Construction of this is discussed below.

Calibration Data—These data were determined with pure, natural (dextro) β -, γ -, and δ -tocopherols. Table I gives Beckman spectrophotometer density readings at 410 $m\mu$. Nitroso- γ -tocopherol values, obtained in triplicate, and nitroso- β -tocopherol, in duplicate, show reproducible values and a straight line relationship between color and concentration determined over a wide range. However, δ -tocopherol appears to give more variable results, especially at higher levels of concentration. For this reason the Evelyn photometer, which is more sensitive than the Beckman, may be the preferable instrument. It is also simpler and more convenient to use. Values obtained with the Evelyn photometer for the three tocopherols, with Filter 400, are given in Table II. Mean K values of 1.57, 1.13, and 1.10 were found for β -, γ -, and δ -tocopherols, respectively. Combined γ - and δ -tocopherols in a mixture (as in soy bean oil) can be determined with a maximum deviation from the true value of ± 7 per cent with the use of a mean K value, *e.g.*, 1.12.

EXPERIMENTAL

Illumination was found to be a most important factor in the method. Direct daylight has a destructive effect on the nitrosotocopherols, but no trouble is experienced if artificial light is used.

The pH of the nitrosation reaction mixture was determined with a glass electrode (Beckman) pH meter. The mixture composed of 5 ml. of ethanol + 0.2 ml. of glacial acetic acid + 3 ml. of 2 per cent sodium nitrite had a "pH" of 4.7. Addition of 2 ml. of 20 per cent potassium hydroxide increased the pH to more than 13. The alkalization step was found necessary to stabilize the color after the subsequent extraction into Skellysolve H.

The time of nitrosation was varied and the results compared with a 1 minute time interval. A 10 minute period allows destruction or alteration of the nitrosotocopherol as shown by the absorption spectrum. Fig. 3 shows spectra of nitroso- δ -tocopherol prepared with a 10 minute and with a 1 minute time of nitrosation. In some cases a 2 minute nitrosation period may be of value, particularly in assaying for δ -tocopherol alone. The calibration curve is then more nearly a straight line in the higher concentration range. The β - and γ -tocopherol calibration curves prepared with a 2 minute interval appear to change somewhat in slope but are still linear.

The precision of assay of solutions of the tocopherols is good. For example, seven single assays of 0.500 mg. samples of tocopherol, each done on different days, gave K values (Evelyn photometer) of 1.56 ± 0.017 (standard deviation), 1.14 ± 0.019 , and 1.10 ± 0.054 for β -, γ -, and δ -tocopherols, respectively. For a solution of unknown potency (com-

TABLE I

Calibration Data for Nitrosotocopherols with Beckman Spectrophotometer at 410 m μ

Kind of tocopherol	Concentration, mg. of tocopherol per 12 ml. Skellysolve H	Density at 410 m μ		$E_{1\text{ cm.}}^{1\%}$ of mean values
		Individual values	Mean value	
β -	0.500	0.174		
		0.169	0.172	41.3
	1.00	0.351		
		0.349	0.350	42.0
	1.50	0.532		
		0.529	0.531	42.5
	2.00	0.719		
		0.698	0.709	42.5
	2.50	0.885		
		0.892	0.889	42.6 (42.2)*
γ -	0.500	0.248		
		0.219		
		0.228	0.232	55.7
	1.00	0.482		
		0.468		
		0.471	0.474	56.9
	1.50	0.732		
		0.721		
		0.730	0.723	58.2
	2.00	0.990		
		1.00		
		1.00	0.997	59.8
	2.50	1.21		
		1.21		
		1.21	1.21	58.1 (57.7)*
δ -	0.500	0.255		
		0.272		
		0.271		
		0.257		
		0.266	0.264	63.3
	1.00	0.500		
		0.538		
		0.535		
		0.484		
		0.489	0.509	61.0
	1.50	0.690		
		0.792		
		0.752		
		0.698		
		0.729	0.732	58.6

TABLE I—*Concluded*

Kind of tocopherol	Concentration, mg of tocopherol per 12 ml. Skellysolve H	Density at 410 $m\mu$		E_1^1 % of mean values
		Individual values	Mean value	
	2.00	0.865 1.03 0.985 0.845 0.874	0.920	55.2
	2.50	1.07 1.24 1.18 1.04 1.08		
			1.12	53.7 (58.4)*

* Mean for group

mercial distilled vitamin E concentrate) eight consecutive assays for total non- α -tocopherols gave 13.5 ± 0.16 (standard deviation) per cent of oil.

The color of the Skellysolve H solution of nitrosotocopherol is stable for periods of several hours up to a day or more in the absence of daylight. All operations should be carried out under subdued artificial illumination.

The effect of oil on the "nitroso" assay was studied in a few experiments. Olive oil, which contains negligible amounts of non- α -tocopherols, was added to varying amounts of the pure tocopherols and the resultant color compared with control solutions of the tocopherols alone. Maximum deviation of ± 10 per cent was noted when 20 mg. of oil were added to 0.5 mg. of tocopherol. This corresponds to a tocopherol concentration in oil of only 2.5 per cent. The olive oil gave no color in the nitrosation assay. At higher concentrations of tocopherol in oil, the effect noticed is much less.

Chromatographic Separation of Nitrosotocopherols

Into a glass-stoppered cylinder pipette at least 12 ml. of the Skellysolve H extract of nitrosotocopherols, prepared as directed. For this purpose combine duplicate assays, if necessary. Add an equal volume of distilled water and a pinch of anhydrous sodium sulfate. Stopper and shake the flask for 30 seconds in order to wash out the small amount of ethanol which is dissolved in the Skellysolve H layer.

Pack a chromatograph column 15×1.3 cm. with ZnCO_3 -Celite (70:30),²

² The zinc carbonate used is "purified, precipitated" zinc carbonate obtained from the J. T. Baker Chemical Company, Phillipsburg, New Jersey. Celite is diatomaceous earth obtained from the Johns-Manville Sales Corporation, Celite Division, 220 Delaware Avenue, Buffalo, New York. The grade used is Celite 501.

TABLE II

Calibration Data for Nitrosotocopherols with Evelyn Photometer and Filter 400

Kind of tocopherol	Concentration (C), mg. of tocopherol per 12 ml. Skellysolve H	L_{400} *	$K = \frac{C}{L_{400}}$	Mean K
β -	0.250	0.155	1.61	1.57
	0.500	0.319	1.57	
	0.750	0.475	1.58	
	1.00	0.658	1.52	
	1.25	0.796	1.57	
γ -	0.250	0.227	1.10	1.13
	0.500	0.438	1.14	
	0.750	0.648	1.16	
	1.00	0.912	1.10	
	1.25	1.097	1.14	
δ -	0.250	0.244	1.03	1.10
	0.500	0.472	1.06	
	0.750	0.668	1.12	
	1.00	0.854	1.17	
	1.25	1.112	1.12	

* Each value is the mean of closely agreeing duplicate values.

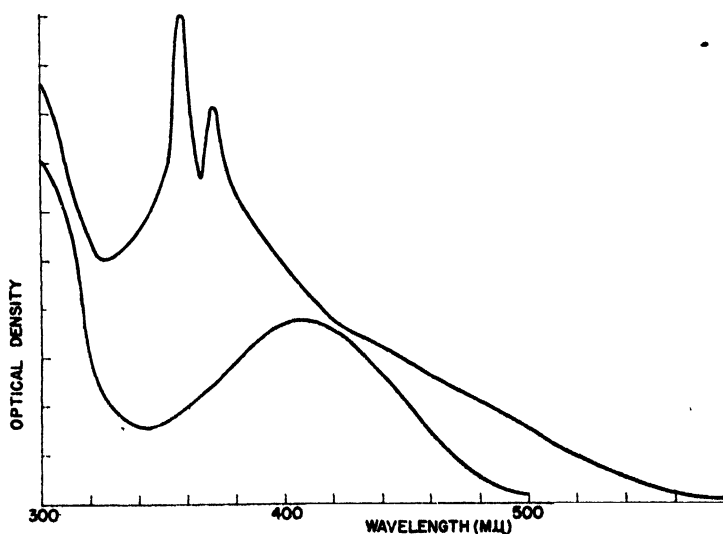


FIG. 3. Effect of time of nitrosation on nitroso- δ -tocopherol. The top curve is of δ -tocopherol allowed to react for 10 minutes with nitrous acid before neutralization and extraction. The lower curve is of nitroso- δ -tocopherol prepared with the usual 1 minute time interval.

with the use of a column having a sintered glass disk set in the bottom to retain the adsorbent. The adsorbent should be added under suction in small portions and each one tamped down firmly.

Wet the column (under suction) with Skellysolve H. Without allowing air to enter the column, pipette 10.0 ml. of the water-washed Skellysolve H solution of the nitrosotocopherols onto the column.

Let this run through almost completely and wash the last traces through with several successive small (less than 1 ml.) quantities of Skellysolve H. Rinse down the walls of the column with a 5 to 10 ml. portion of Skellysolve H.

Without letting the column run dry, develop the chromatogram with pure benzene. Continue washing with benzene until the nitroso- γ - and nitroso- β -tocopherol rings (lowest and middle, respectively) have been washed through into separate receivers. Elute the nitroso- δ -tocopherol ring (or rings) with 1:1 ether-ethanol. Collect in one container. (Alternatively, the column can be sucked fairly dry and the rings separately extruded with a spatula, put immediately into a flask containing ethanol-ether, and these filtered through filter paper containing a layer of Celite to remove the adsorbent.)

Evaporate all three eluates just to dryness under a stream of nitrogen, taking care not to overheat the residue. Take up each residue in 10.0 ml. of Skellysolve H. Let cool to room temperature and transfer to Evelyn photometer tubes. Read with Filter 400 against a pure solvent (Skellysolve H) blank set at 100. Evaluate the results as before, using the respective K values for nitroso- β -, γ -, and δ -tocopherols. If desired, Beckman spectrophotometer density readings at 410 $m\mu$ can be used instead.

EXPERIMENTAL

The Skellysolve H solution of the nitrosotocopherols as prepared in the assay method is ready for chromatographic adsorption after a single distilled water wash. This removes traces of alcohol which are contained in the Skellysolve H layer and which would prevent complete adsorption otherwise.

For developing the chromatogram, benzene was found most suitable of several solvents tried. The nitrosotocopherols then appear as separate bands on the column, being in order from top to bottom the derivatives of δ -tocopherol (two rings), β -tocopherol, and γ -tocopherol. Their colors differ sufficiently to aid in their identification. Nitroso- γ -tocopherol is rosy red, nitroso- β -tocopherol is brownish red, while nitroso- δ -tocopherol is orange-red.

Benzene is also used to elute the two lower bands on the column. A mixture of ethanol-ether elutes the combined nitroso- δ -tocopherol bands.

Commercial c.p. benzene is suitable for use provided it contains no non-volatile impurities which absorb light at 410 m μ . Since many samples do, purification may be necessary. This can be done by redistilling, taking a middle portion, and adding water to approximately one-half saturation.

Glass wool in the adsorption column showed a tendency to hold up the nitrosotocopherols during elution. Hence a column with an inset sintered glass plate was constructed and has proved convenient. A column-receiver assembly with interchangeable standard taper joints has been found useful, especially since contact of the organic solvents with rubber stoppers must be avoided owing to possible contamination.

Results

Synthetic mixtures of β -, γ -, and δ -tocopherols in the presence of α -tocopherol have been assayed and separated chromatographically. Data are shown in Table III. Each mixture contained 0.500 mg. each of the four natural tocopherols. The separated nitrosotocopherols were evaluated by Beckman spectrophotometer and by Evelyn photometer readings. Quantitative recovery of these fractions is shown. The higher recoveries of nitroso- β -tocopherol are not explained at present. They are not due to alteration of nitroso- β -tocopherol on the column, since on chromatography of it alone recoveries of 94.5, 96.5, and 100.0 per cent were obtained.

Synthetic mixtures of γ - and δ -tocopherols have been nitrosated and separated. For these a shorter column of adsorbent (7 cm.) suffices, since the middle band (nitroso- β -tocopherol) of the three component mixture is not present and less development of the chromatogram is required. Four solutions containing 0.500 mg. each of pure natural γ - and δ -tocopherols were resolved. Data are given in Table IV. Recoveries of the eight fractions ranged from 95 to 108 per cent with a mean of 100 per cent. Recoveries of the total tocopherols in the mixtures varied from 101 to 108 per cent with a mean of 103 per cent.

A series of γ - and δ -tocopherol mixtures of natural origin was assayed, which consisted of eleven fractions of an analytical molecular distillation of soy bean oil. Recoveries of the eluate fractions, as compared to the original unchromatographed material, were for the entire series within the range 97.7 to 105.5 per cent. Mean recovery was 101.0 ± 2.4 per cent (standard deviation). The successive fractions showed a smooth progression in relative concentrations of δ - and γ -tocopherols such as would be expected from their relative molecular weights. Elimination maxima of the two tocopherols were found to differ by about 4°.

Vegetable oils can be assayed by the method following preliminary concentration, preferably by laboratory molecular distillation (3). If

TABLE III

Chromatographic Separation of Synthetic Mixtures of β -, γ -, and δ -Tocopherols after Nitrosation

Experiment No.*	Band position on chromatogram	Nitroso derivative of	Controls	Eluates	Recovery, $\frac{D_{eluate}}{D_{control}} \times 100$
			Beckman, density at 410 m μ		
1	Top	δ -Tocopherol	0.253	0.266	<i>per cent</i>
	Middle	β -Tocopherol	0.175	0.191	105
	Bottom	γ -Tocopherol	0.230	0.233	109
					101
Total..			0.658	0.690	105
2	Top	δ -Tocopherol	Evelyn, L_{880} values		$\frac{L_{eluate}}{L_{control}} \times 100$
	Middle	β -Tocopherol	0.469	0.469	100
	Bottom	γ -Tocopherol	0.319	0.357	112
			0.438	0.441	101
Total.....			1.226	1.267	104

* In both experiments a mixture of 0.500 mg. each of α -, β -, γ -, and δ -tocopherols was nitrosated and chromatographed according to procedures given. Control solutions were nitrosated separately and not chromatographed.

TABLE IV

Chromatographic Separation of Replicate Synthetic Mixtures of γ - and δ -Tocopherols after Nitrosation

Mixture No.*	Results by "nitroso" assay		
	Tocopherols in original mixture	Recovery of separate tocopherols in eluates, per cent of controls	Recovery of total tocopherols, per cent of original mixture
	mg.		
1	0.993	(γ -) 104 (δ -) 97	104
2	1.01	(γ -) 108 (δ -) 101	108
3	0.993	(γ -) 98 (δ -) 95	100
4	1.01	(γ -) 97 (δ -) 101	101
Mean re- covery, %		100	103

* Each mixture contained 0.500 mg. each of γ - and δ -tocopherols and was nitrosated and chromatographed according to the procedure given. Controls were unchromatographed nitrosated solutions of γ - or δ -tocopherol of equal concentration.

the distillate contains an excess of fatty acids, they can be washed out of ether solution by dilute alkali. Yellow color in the distillate due to carotenoids is conveniently removed by hydrogenation (4).

The technique described in this paper is useful as a qualitative test for tocopherols in oils. For example, wheat germ oil was found to give a yellow color on nitrosation (of a hydrogenated molecular distillate). The Beckman spectrum indicated it to be a nitrosotocopherol. On chromatog-

TABLE V

Content of Individual Tocopherols of Vegetable Oils and Commercial Vitamin E Concentrates

Sample		Tocopherol content, mg. per gm.				
Kind of oil	Source	Total	β -	γ -	δ -	α -
Corn salad oil	Mazola oil, purchased locally	1.02		0.894		0.126
Cottonseed salad oil	Wesson oil, purchased locally	0.870		0.377		0.493
Soy bean salad oil	Purchased from Blanton Company, St. Louis	0.740		0.418	0.226	0.096
Wheat germ oil*	Merit wheat germ oil	2.68	1.07			1.61
Distilled concentrate of <i>d</i> - α -tocopheryl acetate†	Distillation Products, Type VI, pilot plant sample	366	96.4	9.2	26.4	234‡
Distilled concentrate of <i>d</i> - α -tocopherol	Distillation Products, Type V, pilot plant sample	339	61.4	18.6	15.0*	244

* The sample was bioassayed by Miss M. Ludwig of our laboratories and found to have a potency equivalent to 2.09 mg. per gm. of *d*- α -tocopherol. Assuming that the 1.07 mg. of β -tocopherol is equivalent in potency to 0.36 mg. of α -tocopherol (5), the total *d*- α -tocopherol equivalence estimated from the chemical analysis is (0.36 + 1.61) 1.97 mg. per gm.

† Assayed after preliminary saponification.

‡ This corresponds to 257 mg. of *d*- α -tocopheryl acetate.

raphy, a single red band showed the presence of only one non- α -tocopherol. Identity of this with β -tocopherol was shown by augmentation of the one band when β -tocopherol was added to a wheat germ oil assay mixture, which was then nitrosated and chromatographed. A similar experiment with corn oil showed only the presence of γ -tocopherol. Cottonseed oil gave anomalous behavior. The nitrosotocopherol split into two rings on the column which recombined on continued development with benzene to give a homogeneous band. Added δ -nitrosotocopherol did not augment

either part of the split band, showing absence of it in the cottonseed oil in amounts detectable by the method.

Quantitative data on tocopherols in a few vegetable oils are given in Table V. The method is now in general use in the laboratories of Distillation Products for the assay of commercial concentrates which are mixtures of α -, β -, γ -, and δ -tocopherols. A few results are also shown in Table V. Since tocopherol esters do not give nitroso derivatives in the assay proce-

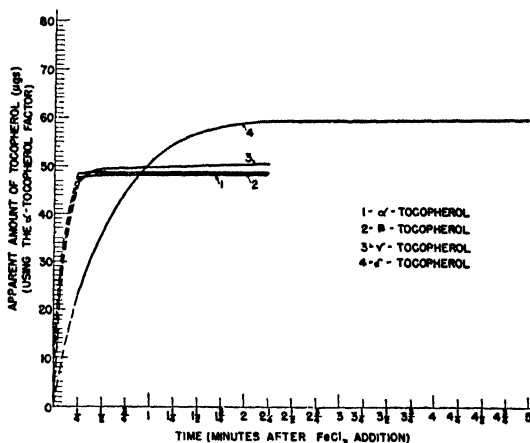


FIG. 4. Time curves for pure natural α -, β -, γ -, and δ -tocopherols in the Emmerie and Engel reaction according to the technique of Quaife and Harris. Each curve represents the means of quadruplicate values which agreed very closely. Each Evelyn photometer tube contained 8.0 ml. of ethanol solution of 48.0 γ of tocopherol plus 1.0 ml. each of α , α' -dipyridyl and ferric chloride reagents. The ordinate values were calculated with the use of a factor determined, on repeated calibration, for the 15 second reading with pure natural d - α -tocopherol. The abscissa values refer to the time after addition of the last drop of ferric chloride reagent. The tocopherols were samples of the pure natural d forms.

ture, it is necessary first to saponify samples containing them (e.g., Distillation Products concentrate, Type VI, for which values are given in Table V).

In all cases α -tocopherol was determined by difference between total tocopherols and non- α -tocopherols. The former were measured by the Emmerie and Engel reaction (6), modified to take account of the very different behavior of δ -tocopherol from that of the α , β , and γ forms. One such modification is given by Stern *et al.* (7). Another uses the Quaife and Harris technique (8) except that a 50 second reading is used in place of a 15 second one. Calculation, as before, is made with a K determined by calibration with pure natural α -tocopherol, with a 15 second reading. Time curves of the four natural tocopherols for the reaction are given in

Fig. 4. They show equal color development per unit weight (within limits of ± 3 per cent) at 50 seconds.

SUMMARY

The nitrosotocopherols of β -, γ -, and δ -tocopherols, formed by treatment of the tocopherols with nitrous acid, can be separated by a simple chromatographic step and evaluated photometrically.

This is the basis of a method for the specific determination of β -, γ -, and δ -tocopherols in admixture and in the presence of α -tocopherol. The latter is determined by difference from the Emmerie and Engel value for total tocopherols, modified to take account of the varying behavior of the tocopherols. The methods are given in detail.

Separation of synthetic mixtures of the tocopherols and assay of vegetable oils and commercial vitamin E concentrates are described.

Use of the method as a qualitative test for tocopherols in natural materials is suggested and illustrated.

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THE ASSAY OF ANIMAL TISSUES FOR RESPIRATORY ENZYMES

VII. OXALACETIC ACID OXIDATION AND THE COUPLED PHOSPHORYLATIONS IN ISOTONIC HOMOGENATES*

By VAN R. POTTER, G. A. LePAGE, AND H. L. KLUG

(From the McArdle Memorial Laboratory, Medical School, University of Wisconsin, Madison)

(Received for publication, April 8, 1948)

Previous reports from this laboratory have shown that oxidative phosphorylation could be demonstrated in a variety of tissues with the use of water homogenates in reaction mixtures that include fluoride (1-3). Attempts to eliminate the fluoride were partially successful (3) but were handicapped by the use of water homogenates, as will be shown below.

Water homogenates had been successfully employed in previous studies on cytochrome oxidase and the succinic oxidase system (4), on the malic system (5), and on the glycolytic system (6, 7) and indeed appeared to be better than isotonic homogenates in most respects.

However, Lehninger (8) showed that isotonic homogenates are superior to water homogenates in the case of octanoate oxidation, and our experiments on this system (9, 10) as well as those of Cohen and associates (11, 12) on other systems further emphasized the importance of the isotonic homogenate for oxidative systems involving adenosine triphosphate (ATP). Although we had suggested that the isotonic homogenates contained a large proportion of "whole" cells on the basis of "cytolysis quotients," it is now clear that the data which led to this conclusion can be explained in terms of the structural integrity of the mitochondria contained in the cells, rather than in terms of the integrity of the cells (13). Isotonic homogenates therefore may contain much higher proportions of disrupted cells than we had concluded earlier (9, 10). What is perhaps more important is the fact that the isotonic homogenates appear to contain cytoplasmic particles that are functionally greatly different from the particles found in water homogenates. Moreover, the enzymes in these particles appear relatively more accessible to many metabolites than they are in tissue slices (*cf.* Cohen and Hayano (11)).

In view of the importance of isotonic media in systems that are correlated with ATP synthesis (8-13) it became necessary to reexamine the

*This work was aided by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council and by a grant from the Jonathan Bowman Fund for Cancer Research.

systems previously used for the study of oxidative phosphorylation (1-3) since these studies had been made with water homogenates. The present report deals with the phosphorylation and oxygen uptake that result when oxalacetic acid is used as a substrate, since it was found that this system, in contrast to the systems involved in glycolysis (7), succinate oxidation (9), and malate oxidation (5), is extremely sensitive to the tonicity of the homogenization medium.

The mechanism of oxidation of oxalacetic acid remains unknown in spite of scores of publications that have considered various aspects of it. Presumably we are here dealing with one of the more sensitive components of the Krebs cycle of oxidations (see (14)), since the system fails under conditions (4, 5) that permit the oxidation of succinic and malic acids. Since the oxygen uptake observed is several fold greater than the one-stage oxidation would require, it appears that in this system the oxidation is carried approximately to completion; that is, to CO_2 and water via the Krebs cycle as will be shown later. Since in most cases the results cannot be duplicated by using pyruvate instead of oxalacetate, it seems likely that the oxidation of oxalacetate must be preceded by the Krebs condensation, but it is as yet unknown whether this reaction involves the condensation of oxalacetic acid with a C_2 or a C_3 compound. Following the conventional nomenclature, we shall refer to the enzymes involved as the "oxalacetic oxidase system" since more than one enzyme seems likely to be involved. The enzyme system is quite likely to be the same one that Green has referred to as "cyclophorase" (15).

In this paper, the effect of variations in the homogenate will be reported, including variations in the preparation and handling of the homogenate as well as variations in the type of tissue used. The effect of variations in the reaction mixture will be reported in a separate paper.¹ A preliminary report on the failure of tumor tissue to oxidize oxalacetic acid under the conditions herein described has been presented by Potter (16).

EXPERIMENTAL

Oxidation Studies—Oxygen uptake was measured in a conventional Warburg apparatus at 38° after allowing 10 minutes for equilibration. The final volume of the reaction mixtures was 3.0 ml. in all cases and the molarity of the added reagents is given with the volume added per flask. When the stated molarity refers to the final reaction mixture, it is so designated.

Phosphorylation Studies—When phosphate fractionations were carried out, the reaction was stopped by the addition of 2 ml. of 17.5 per cent cold trichloroacetic acid, as in the earlier work (1-3). The precipitated

¹ Potter, V. R., Pardee, A. B., and Lyle, G. G., *J. Biol. Chem.*, in press.

protein was removed by centrifuging and 0.1 ml. aliquots of the supernatant fluid were used for the determination of "true" inorganic phosphate by the method of Lowry and Lopez² (3), and for total acid-soluble phosphate (17). The remainder of the supernatant fluid was fractionated in the cold as follows: A 3.5 ml. aliquot was carefully neutralized with about 1.5 ml. of 1.0 N KOH to pH 8.2 with dilute phenolphthalein in the solution. Inorganic phosphate and ATP were precipitated by adding 0.15 ml. of 1 M Ba(OAc)₂. After centrifuging, the supernatant was poured into a tube containing 0.37 ml. of 1 N H₂SO₄, the BaSO₄ was centrifuged, and the supernatant was analyzed for phosphocreatine by the Fiske-Subbarow method (1). The precipitate containing the inorganic phosphate and ATP was taken up in 2.0 ml. of water and 1.0 ml. of 0.1 N H₂SO₄ to remove Ba. The supernatant was saved and the precipitate was treated a second time with 0.5 ml. of acidified water. The washing was combined with the supernatant and treated with 0.1 ml. of concentrated NH₄OH and 0.10 ml. of magnesia mixture (17) to precipitate inorganic phosphate. The precipitate was separated by centrifuging and the supernatant (ATP fraction) was poured into a tube containing 0.30 ml. of glacial acetic acid to give a pH of 4.5 to 4.8, any of the precipitated inorganic phosphate that may have been included being dissolved in the supernatant. Aliquots of 0.3 ml. of the ATP fraction were then analyzed for inorganic phosphate and 7 minute-hydrolyzable phosphate (17) in order to obtain the $\Delta 7$ phosphorus, which was taken as a measure of ATP. The removal of the bulk of the inorganic phosphate by the magnesia mixture increases the accuracy of the $\Delta 7$ measurement. In addition, 0.3 ml. aliquots of the ATP fraction were analyzed for ribose (17), the amounts of which paralleled the amounts of $\Delta 7$ phosphorus.

The corrected inorganic phosphate uptake was calculated by subtracting the changes in the sum of the $\Delta 7$ P, phosphocreatine P, and true inorganic phosphate from the true inorganic phosphate. This amounts to subtracting the observed changes in the sum of the phosphocreatine and $\Delta 7$ P from the observed changes in the true inorganic phosphate (see "Discussion").

The phosphorylation experiments usually consisted of sixteen flasks which contained groups of four reaction mixtures that were stopped after different periods of time. The sixteen filtrates were then analyzed simultaneously, all the centrifuging and fractionation being carried out in the cold.

² In the original Lowry-Lopez method, interference by some tissue extracts was noted. We have found that glutathione retards the rate of color development markedly. Thus 60 γ of GSH reduced the color by about 60 per cent at 5 minutes, but the value attained in 30 minutes was equal to that in the control tube. With the amounts of tissue used in the present experiments, no interference by GSH occurs.

Homogenates—Two types of homogenates were used in the experiments described in this paper. Each type was prepared with 9 volumes of fluid per fresh weight of tissue. The "water homogenates" were prepared with distilled water and the "isotonic homogenates" were prepared with isotonic KCl (1.15 per cent) to which had been added 8 ml. of 0.04 M KHCO_3 per liter, to give a pH of 7.5 to 7.8. The tissues were placed in the calculated amounts of medium before the homogenization, which was carried out in the cold, with close fitting glass homogenizers of the Potter-Elvehjem type (18).

Substrates—The substrates employed were oxalacetic and pyruvic acids. Mixtures of oxalacetic acid and pyruvic acid in general gave results almost identical with oxalacetate alone, but superior to pyruvate alone. Since the decarboxylation of oxalacetic acid results in the disappearance of 1 acid equivalent, the neutralization of oxalacetic was not carried to completion. Empirical tests indicated that an amount of alkali equal to the acid groups present after a 50 per cent conversion of oxalacetic to pyruvic acid was near the optimum. The oxalacetic acid was therefore neutralized by adding 0.02 M K_2CO_3 to solid oxalacetic acid to give a final oxalacetic acid concentration of 0.0267 M. The acidic solution was then aerated to drive off the excess CO_2 . Since oxalacetic acid is not stable, solutions were prepared only 10 to 15 minutes before being pipetted.

Components of "Complete" System—The reaction mixture that has been employed for the present series of experiments differed from the previous mixture (3) mainly in the fact that potassium salts were used throughout and fluoride was omitted. The following components were used in the complete system in the present study: water to make 3.0 ml. of final volume, 0.4 ml. of 0.5 M KCl, 0.1 ml. of 0.1 M MgCl_2 , 0.1 ml. of 0.1 M K phosphate ($\text{KH}_2\text{PO}_4 + \text{KOH}$ to pH 7.4), 0.1 ml. of 4×10^{-4} M cytochrome *c*, 0.2 ml. of 0.01 M K ATP, 0.1 ml. of K DPN¹ $\approx$ 660 γ of DPN, 0.5 ml. of isotonic KCl if water homogenates were used or an amount to make 0.5 ml. if less than 0.5 ml. of isotonic homogenate was used, 0.3 ml. of 0.0267 M oxalacetate (see above), and 0.1 to 0.5 ml. of cold 10 per cent homogenate. When creatine was added, as in the case of the phosphorylation studies, 30 mg. of the hydrate were placed in the flask before the other additions, and the 0.5 M KCl was decreased from 0.4 to 0.2 ml. Flasks without side arms were used, and were kept in ice until the homogenate had been added. It is possible to combine the KCl, MgCl_2 , phosphate, and cytochrome into a stock solution if most of the water addition is included. The ATP and DPN can also be combined into one solution. These two mixtures can be kept in the refrigerator for at least a week. The effect of a lack of DPN

¹ DPN = diphosphopyridine nucleotide, coenzyme I.

and cytochrome *c* is not striking, but they were originally added on the basis of the known need for these substances in the malic system (5), and the total operation of the Krebs cycle was considered likely in this system. Furthermore while some tissues might not require additional cytochrome *c* and DPN, others (such as tumors) might be expected to be deficient in these accessory compounds. In the work described below, various procedures that were known to result in the loss of the effective concentration of cytochrome *c* in the cytoplasmic particles (13) were tested for their effects on the oxalacetic system, and, since the inclusion of DPN and cytochrome *c* in the reaction mixture has never given any evidence that they are detrimental, they were routinely included in these experiments. The effect of their omission and of other variations in the reaction mixture will be given in the next paper.¹

Observations with Various Tissues—Experiments with various tissues were performed with 50 mg. of tissue per flask, added in the form of a 10 per cent isotonic KCl homogenate, after preliminary experiments had indicated that this level of tissue was in the range of proportionality. The results are shown in Fig. 1. Each curve represents the average of four to six samples of tissue, each of which was run in duplicate or triplicate. The normal tissues were taken from white rats from the stock colony; no normal tissues from tumor-bearing animals have been studied. The hepatoma samples were primary tumors obtained through the courtesy of Dr. J. A. Miller and Dr. E. C. Miller; the Walker 256 and the Flexner-Jobling carcinoma were transplantable tumors provided by Mr. B. E. Kline. All tumors were non-necrotic and in good condition.

The data in Fig. 1 show that there is considerable variation among the tissues, not only in terms of the initial rate of oxidation but also in terms of the way in which the rate changes with time. In all tissues there is a decline in the rate of uptake which does not appear to be the result of substrate depletion, but is probably caused by a decrease in the amount of active enzyme-substrate complex. This decline is slowest in liver but occurs in all of the tissues studied. In the case of the tumor samples, the rate of uptake was negligible, and was no greater than could be obtained in the absence of added oxalacetate (not shown in Fig. 1). The no substrate rate of oxidation was very low in all of the normal tissues except liver, in which the initial rate of oxidation was appreciable during the first 30 or 40 minutes (40 to 50 microliters of O₂ per 50 mg. of liver) but declined rapidly thereafter. It seems possible that this endogenous respiration in liver may be related to the fact that the enzyme activity in liver does not decline as rapidly as in the other tissues either during the reaction (Fig. 1) or in the homogenate. The declining rates in the normal tissues suggested that the observations in the case of the tumor samples

might be due to the lability of the enzyme system, and numerous studies have been carried out to ascertain the factors involved in the breakdown of the enzymes involved in the reaction.

Enzyme Breakdown in Homogenates and in Situ—The standard procedure in the preparation of homogenates has been to kill the animal by decapitation, and to remove the tissue quickly, placing it in cold isotonic KCl surrounded by ice. The homogenate was then prepared in the cold and kept in ice until it was used, which was normally within about 10 minutes. Fig. 2 shows the effect of storing the homogenates at 0° for various periods of time. The experiment was carried out with five different tissues

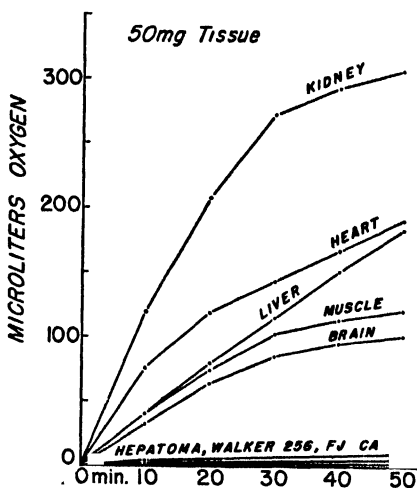


FIG. 1. Oxygen uptake by isotonic KCl homogenates of various tissues, 50 mg. of fresh tissue per flask. "Complete" reaction mixture described in the text with creatine omitted.

from three to six animals for each kind of tissue. Each homogenate was tested in duplicate or triplicate after 0, 6, and 24 hours at 0°. The results are the averages of all the determinations. The tissues all showed very little decline in activity during the first 6 hours, but kidney, brain, and skeletal muscle showed considerable decline after standing overnight at 0°. The activity in heart and liver homogenates was maintained for 24 hours. There was slight variation among the various samples except in the kidney homogenates stored 24 hours, in which the per cent loss varied considerably. The storage at 0° is comparable to a limited dialysis; diffusible substances may escape from the nuclei and mitochondria and attain a 10-fold dilution, since a 10 per cent homogenate is employed. The loss in activity may be comparable to that obtained by Gibson and Long

(19) in the case of pyruvic oxidase in minced heart muscle upon dialysis. They found that magnesium ions prevented the inactivation.

On the other hand, the inactivation occurs in the intact tissue *in situ* in which magnesium is probably adequate (Fig. 3). For these experiments the animals were decapitated and placed in a warm room at 38°. In the case of skeletal muscle, brain, and kidney one sample could be taken from the right side and a second sample taken from the left side after a period at 38°. The second half of the brain was placed inside the abdominal cavity to avoid drying. Single samples of heart were taken,

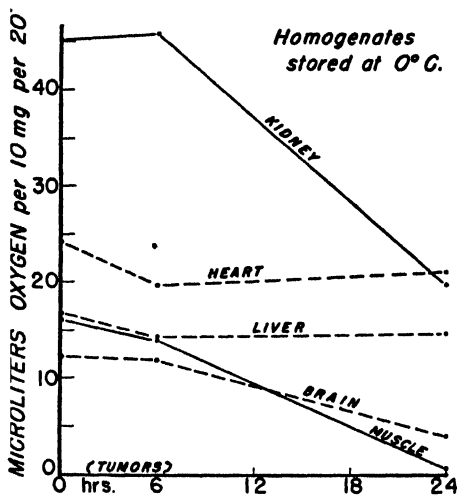


FIG. 2.

FIG. 2. Oxygen uptake by isotonic KCl homogenates of various tissues following storage at 0° for 6 to 24 hours. Conditions as in Fig. 1. Experiments with tumors not done because of low values at zero time.

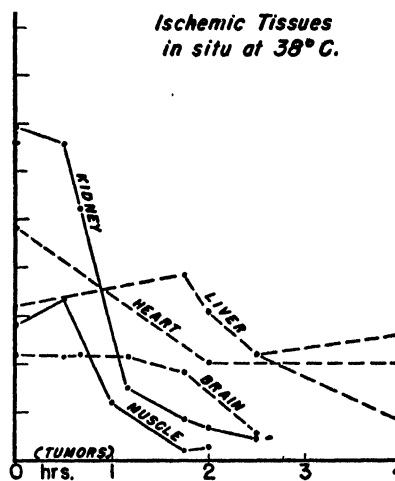


FIG. 3.

FIG. 3. Oxygen uptake by isotonic KCl homogenates of various tissues taken from decapitated rats after various periods at 38°. Conditions as in Fig. 1. Experiments with tumors not done because of low values at zero time.

while several samples of liver could be obtained. In the case of pairs of samples, the first was not always taken at zero time. Each point on the curves represents four to six determinations in duplicate. The results show that during the first 30 minutes there is essentially no loss in enzyme activity, but that following this period the loss is quite variable among the different tissues. The rapid loss in activity in the muscle and kidney is of considerable physiological interest, since it correlates to some extent with the ability of these tissues to return to normal following ischemia (20, 21).

The results of both studies indicate that if tissues are removed within 30

minutes and homogenized in the cold they may be used for the study of oxalacetate oxidation, or, if removed at once and homogenized, the homogenate may be stored at 0° up to 6 hours.⁴ In these studies it was found that frequently the activity of an aged preparation was normal initially but that the decline in rate was more pronounced than in the fresh preparation. Neither experiment was done with tumors because the activity in fresh preparations was too low to measure.

Enzyme Breakdown in Water Homogenates and at 38°—It was stated earlier that water homogenates of liver would not oxidize oxalacetic acid (2, 3). Fig. 4 shows the results of studies in which aliquots of various tissues were homogenized in water and in isotonic KCl and aliquots of each homogenate were incubated for 15 minutes at 38°. As was shown in Fig. 2, an aliquot of the homogenate could be held at 0° for more than 15 minutes without loss in activity while another aliquot was being incubated at 38°. The results of these experiments could not be given in terms of the oxygen uptake per 20 minutes, and were plotted as total oxygen uptake *versus* time. The reason for this is apparent in the case of the water homogenates of kidney (Fig. 4). In these samples, the initial rate of oxidation was near normal, but after about 20 minutes the rate suddenly fell to a very low level in comparison to the rate in the KCl homogenate. In the case of the liver, homogenization in water gave almost complete inactivation of the enzyme system even in the case of the initial rate, while in brain homogenization in water gave an effect that was intermediate between what was found in kidney and in liver.

The effect of incubating these homogenates at 38° is also shown in Fig. 4. In 15 minutes, the destruction of activity was essentially complete in both types of kidney homogenate and in the KCl homogenate of brain. In liver, on the other hand, the activity was completely unaffected in the case of the KCl homogenate, incubated at 38°, while the water homogenate was already inactive. The greater stability of the liver KCl homogenate at 38° parallels its stability at 0° and may be related to its higher endogenous respiration.

None of these procedures affects the ability of homogenates to oxidize succinate or malate (1, 5) but profoundly affects the ability to promote oxidative phosphorylation (1). The following experiments indicate that the ability of a homogenate to oxidize oxalacetic acid is a reflection of its ATP balance, and that the simple measurement of rate and duration of oxidation (Figs. 1 to 4), unlike the measurements of succinate or malate

⁴ Parallel experiments on these tissues showed that freezing the tissues or the homogenates resulted in the loss of the ability to oxidize oxalacetate. These considerations are of some importance if large numbers of tissue samples are to be studied.

oxidation, provides a measure of the capacity of the homogenate to maintain the ATP reservoir by oxidative phosphorylation.

Phosphate Balance Studies—In the absence of fluoride, the breakdown of ATP is much more rapid than in its presence, but even in the absence of fluoride a temporary maintenance of ATP in terms of a constant level of inorganic phosphate was observed in rat kidney (3). Further experiments were devised to show the complex nature of the factors affecting the level of true inorganic phosphate in the reaction mixtures described above.

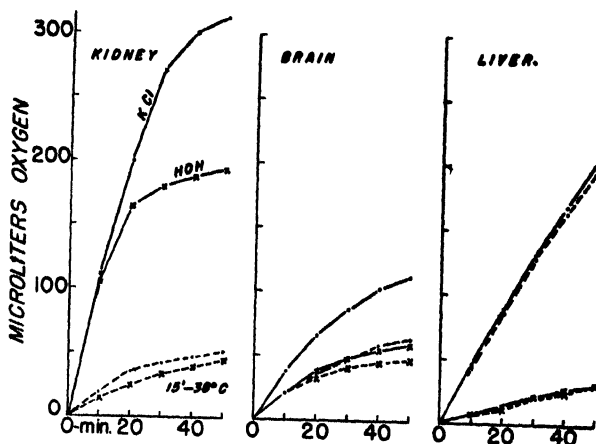


FIG. 4. Oxygen uptake by isotonic KCl homogenates of kidney, brain, and liver compared with water homogenates, showing the effect of 15 minutes incubation at 38° prior to addition to reaction mixture. Test conditions as in Fig. 1. The solid lines represent fresh homogenates; the broken lines represent incubated homogenates. ●, KCl homogenates; ×, water homogenates.

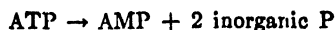
The data obtained with isotonic homogenates in complete systems may first be considered.

Determinations of phosphocreatine (Fig. 5, B) and true inorganic phosphate (Fig. 6, A) showed a lack of parallelism between these two quantities, in contrast to what might have been expected if the ATP system (adenosine triphosphate, adenosine diphosphate (ADP), and adenosine monophosphate (AMP)) was maintained in a steady state, according to the following reactions.

- (1) $\text{ATP} + \text{creatine} \rightleftharpoons \text{ADP} + \text{phosphocreatine}$
- (2) $\text{ADP} + \text{inorganic P} + \text{coupled oxidation} \rightarrow \text{ATP}$

Since the measurements showed more phosphocreatine formation than inorganic phosphate disappearance, it was concluded that the following over-all reaction was probably taking place.

(3)



If reactions (1), (2), and (3) represented the elements of the phosphate balance, the measurement of the $\Delta 7$ phosphorus of the ATP fraction,

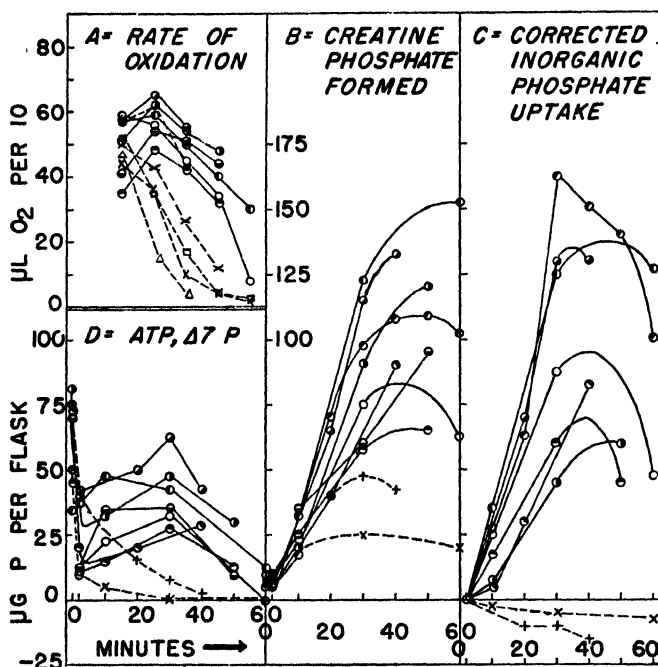


FIG. 5. Phosphate balance studies on homogenates of rat kidney. Conditions as in Fig. 1 except creatine was present. The solid lines represent KCl homogenates; the broken lines represent water homogenates. The points represent matched experiments. Variation among experiments is due in part to animals and in part to the level of ATP used; see Fig. 6, B. A, rate of oxidation; includes a number of experiments on water homogenates in which phosphate studies were not carried out. B, creatine phosphate formation; the experiments with water homogenates were done with different levels of ATP and were compared with isotonic homogenates of the paired kidney. C, corrected inorganic phosphate uptake; data calculated as described in the text, referred to the 2 minute point on the time axis. D, ATP- $\Delta 7$ phosphorus.

together with the true inorganic phosphate and the phosphocreatine, should reveal the change in the dynamic equilibrium of the phosphorylation system: Any increase in phosphocreatine not paralleled by a decrease in inorganic phosphate should be accompanied by an appropriate decrease in the $\Delta 7$ phosphorus of the ATP fraction. The test of this proposition is the addition of the three fractions.

(4) $\text{Phosphocreatine P} + \Delta 7 \text{ P} + \text{inorganic P} = \Sigma \text{P}$

According to the equations (1) to (3), ΣP , which is defined by equation (4), should remain constant. When the actual measurements were carried out, it was found that ΣP increased for a few minutes, then tended to remain constant, and then increased again (Fig. 6, C).

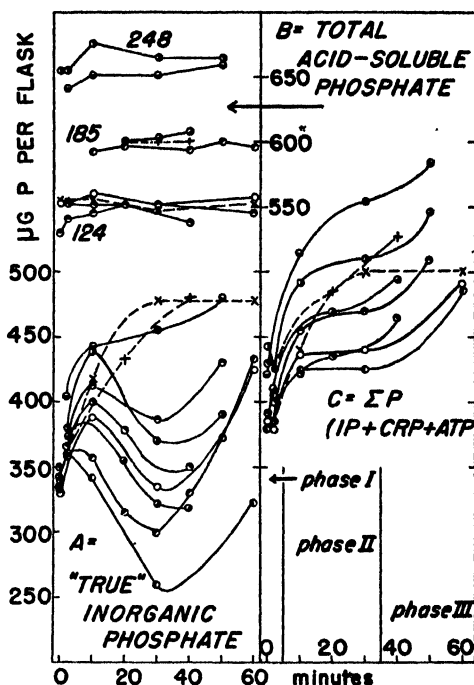
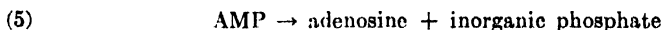


FIG. 6. Phosphate balance studies on homogenates of rat kidney; continuation of data in Fig. 5. A, true inorganic phosphate; B, total acid-soluble phosphate. The figures 124, 185, and 248 refer to the theoretical micrograms of ATP phosphorus added per flask. The point symbols correspond to the similar symbols of Fig. 5. C, sum of the values for the inorganic phosphorus, phosphocreatine, and ATP $\Delta 7$ phosphorus. For discussion of Phases I, II, and III see the text.

Since an increase in ΣP is not explainable solely in terms of equations (1) to (3), other possibilities must be considered. The obvious possibility that nucleoproteins and phospholipids in the homogenate were liberating inorganic phosphate can be eliminated on the basis of the data in Fig. 6, B. The different levels shown represent experiments in which different amounts of ATP were added, but it is clear that in each case the total acid-soluble phosphate did not change significantly during the experimental period. Consequently the increases in ΣP shown in Fig. 6, C must have come from changes within the acid-soluble fraction.

In addition to reaction (3) inorganic phosphate can be formed from the breakdown of AMP⁵ to adenosine by reaction (5):



following the occurrence of reaction (3). With this in mind, the data in Figs. 5 and 6 become much more understandable, and the course of the reaction can be divided into three phases, which have been indicated in Fig. 6, *B* but which can be seen in several of the other measurements in Figs. 5 and 6 as well.

During Phase I, reactions (3) and (5) predominated: ATP broke down to inorganic phosphate (reaction (3)) with little or no transfer to creatine (reaction (1)), while the sudden formation of AMP provided an additional source of inorganic phosphate. During this period oxygen uptake was not being measured, but it appears to have been submaximal, since the first 10 minute period of oxygen uptake measurement usually gave a lower rate than the second 10 minute period. Furthermore, this period, which was only a few minutes long, was one of transitional temperature and probable inadequate oxygenation prior to the beginning of shaking.

With the establishment of oxygenation and the temperature of 38°, the fall in $\Delta 7$ phosphorus ceased and Phase II began. Oxygen uptake accompanied by the disappearance of inorganic phosphate occurred and the $\Delta 7$ phosphate level began to rise and level off at a steady state which was accompanied by the formation of phosphocreatine. The fact that the ΣP tended to remain constant during this period, while the $\Delta 7$ could not regain the original levels, suggests that AMP once dephosphorylated is no longer available for rephosphorylation to ATP.⁶ Studies with AMP substituted for ATP suggested that it does not remain as such in this reaction mixture. In this case the $\Delta 7$ phosphorus was zero at zero time and rose to levels that were less than were attained with equimolar levels of ATP, while the ΣP increased simultaneously. In these experiments, the oxygen uptake, phosphocreatine formation, and corrected inorganic phosphate uptake had about the same values as in parallel experiments with ATP. There appears to be a continuous drain on the ATP system in the form of reaction (5) which is rapidly accelerated when reaction (2) is slowed down and which is minimized by the operation of reaction (2) while oxidation is active. It is likely that the decay of the ATP system is paralleled by a deterioration of the enzyme system involved in oxidative phosphoryla-

⁵ Kornberg and Lindberg (22) have found in kidney a new DPNase that is not inhibited by nicotinamide (*cf.* (3) p. 34). Their enzyme splits AMP from DPN, and the AMP would then be available for reactions (3) and (2) above.

⁶ The possibility that this may be due to the deamination of adenosine to form inosine is currently being investigated.

tion; so that the two processes, while not synonymous, are closely related.

Phase III of the reaction was seen when the rate of oxygen uptake began to fall and the $\Delta 7$ phosphate level began to decrease. At this time the $\Delta 7$ phosphate level fell much more rapidly than the phosphocreatine level, showing that, just as in Phase I, reaction (1) is much slower than reaction (3). Thus in numerous experiments, the $\Delta 7$ phosphorus level could be near zero while the phosphocreatine level was quite high. It is clear of course that the operation of reaction (5) following reaction (3) can prevent the reversal of reaction (1). These data show that the direct dephosphorylation of phosphocreatine (see (23)), if it occurred in these reaction mixtures, was slow enough to permit the occurrence of fairly high levels of phosphocreatine even when the $\Delta 7$ P was being rapidly dissipated. With the loss of the $\Delta 7$ P and the marked decrease in oxidation rate, the Σ P increased toward the level of the total acid-soluble phosphorus, and the true inorganic phosphate level increased. The breakdown of DPN to fragments that included inorganic phosphate may have occurred to some extent in this period;⁵ evidence bearing on this may be seen in the experiments with water homogenates.

In the case of water homogenates of kidney, it will be recalled that oxalacetate oxidation over short periods (Fig. 4; cf. (2, 3)) was possible, but that the reaction soon failed, whereas in the isotonic homogenates it continued for a longer period. The data in Figs. 5 and 6 provided some insight into the reasons for the oxidative failure. Although the initial rate of oxygen uptake was about as good in the water homogenates as in the isotonic homogenates, the ATP was rapidly depleted and was never rebuilt. Some phosphocreatine was formed but it was evidently at the expense of the $\Delta 7$ P, because the corrected inorganic phosphate uptake did not parallel it as in the case of the isotonic homogenates; in fact, the latter data provide the key to the difference between the two types of homogenate. Evidently the water treatment does not destroy the oxidative mechanisms as such but prevents their effective utilization for the maintenance of the ATP system, while the failure to maintain the ATP system is correlated with the decay of the oxidative system.

DISCUSSION

While further discussion of the experimental details is probably not warranted, it is important to note that the use of the isotonic homogenate is highly important in the development of enzyme systems that are capable of utilizing oxidative energy for endergonic syntheses. It would be naive to assume, however, that isotonic KCl represents the ideal homogenization medium, and the importance of further work such as the study of hypertonic sucrose media (13, 24) should be stressed.

The observation (Fig. 1) that isotonic tumor homogenates are unable to oxidize oxalacetate under the conditions described above does not prove that the original tumors are devoid of one or more of the enzymes required for this oxidation, although it seems likely that the tumors must contain very low amounts of the enzyme system. The data could also be obtained if the phosphorylating mechanisms that maintain the oxidative system (and are maintained by it) are inadequate to keep pace with the decay of the oxidative system: the isotonic tumor homogenates that have been studied could have little or none of the oxidative system or they could have a preponderance of the breakdown mechanisms such that no activity could be demonstrated under the conditions employed. In either case, the fact remains that a marked difference between a number of normal tissues and tumors has been demonstrated. One of the differences between tumors and normal tissues is the much higher content *in vivo* of lactic acid in the tumors (25). With this in mind, the experiment shown in Fig. 3 is important because it shows that normal tissues can be left *in situ* for periods long enough to build up a lactic acid content greater than is found in tumors (26) without a decrease in the activity of the oxidizing system.

The inactivity of the tumor homogenates cannot be attributed *solely* to high dephosphorylative mechanisms because homogenates of the same tumors have been shown in this laboratory to maintain their glycolytic activity over long periods of time (27) and to maintain the ATP system by means of the energy of glycolysis (28). The ability of aerobically glycolyzing homogenates (with a maintained ATP system) to metabolize oxalacetic acid is being studied in terms of the hypothesis that in tumors the end-products of glycolysis may be converted to building blocks for growth instead of being burned to CO_2 and water (16).

The establishing of the conditions for handling tissues and homogenates in order to obtain oxalacetate oxidation was a preliminary to further studies designed to establish the experimental basis for the components of the reaction mixture.¹ It is at present impossible to maintain the ATP system in normal tissues for more than limited periods by means of oxidative mechanisms, and the reasons for the decay are still not fully understood. One of the dilemmas that confronts us is the fact that the combustion of the salts of the acids of the Krebs cycle leads to a gradual alkalization of the medium and consequently to an acceleration of ATP breakdown (29). One solution of this problem would be the use of glucose to form lactic acid but this reaction has not been obtained in rat liver and kidney;⁷ cf. also (3).

Another difficulty is our inability to check the breakdown of key com-

¹ LePage, G. A., unpublished work.

ponents of the oxidative phosphorylation system by the action of prosthetic group-removing enzymes, dephosphorylases, deaminases, nucleotidases, etc. Possibly the use of washed residues (8, 12, 15) tends to remove some of these enzymes while retaining the oxidative phosphorylation system in the particulate material, but experiments by Schneider in this laboratory have shown that in the case of liver, at least, the whole homogenate has greater activity than any combination of its sedimentable components.

It is apparent from the data in Figs. 5 and 6 that the study of inorganic phosphate uptake in homogenates cannot be undertaken on the basis of the measurement of inorganic phosphate alone, owing to the large number of reactions that involve the formation and removal of this compound. It is also clear that the occurrence of phases of accelerating and decelerating phosphate uptake must be taken into consideration if exact data on efficiency of phosphorylation mechanisms are to be obtained.

SUMMARY

1. The ability of whole homogenates of rat kidney, heart, liver, skeletal muscle, and brain to oxidize oxalacetate in the presence of magnesium ions, adenosine triphosphate (ATP), inorganic phosphate, diphosphopyridine nucleotide, and cytochrome *c* was determined.

2. Samples of primary hepatoma and Walker 256 and Flexner-Jobling rat tumors took up negligible amounts of oxygen in comparison to the normal tissues studied under the same conditions.

3. Isotonic KCl homogenates were found to give much better results than water homogenates on the basis of comparisons made with kidney, liver, and brain.

4. Activity was measured in KCl homogenates stored for 6 and 24 hours at 0° and in KCl and in water homogenates incubated 15 minutes at 38°, as well as in homogenates from tissues remaining at 38° *in situ* in decapitated animals for 1 to 4 hours. Each tissue gave different results, but there was no loss in activity in any of the tissues in homogenates stored at 0° up to 6 hours or in ischemic tissues *in situ* up to 30 minutes. 15 minutes at 38° inactivated the enzyme in kidney and in brain but not in liver homogenates.

5. Studies of phosphate balance were made on KCl and on water homogenates of rat kidney. Measurements of true inorganic phosphate did not yield figures for phosphate uptake that could be compared with phosphocreatine formation, but, when corrected for changes in $\Delta 7$ phosphorus of ATP and for inorganic phosphate not coming from the $\Delta 7$ fraction, the figures for inorganic phosphate uptake were parallel to the figures for phosphocreatine formation. Homogenization in water results in an increase in dephosphorylation reactions relative to phosphorylation reactions; so

that any systems that are dependent upon the maintenance of the ATP system are weakened or lost.

The authors wish to acknowledge the technical assistance of Mr. John Kallenbach in connection with some of the manometric measurements.

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PARTIAL ACID HYDROLYSATES OF PROTEINS

VI. ASSAY OF LIQUID PROTEIN HYDROLYSATES IN PROTEIN-DEPLETED RATS*

BY DOUGLAS V. FROST AND HARRY R. SANDY

(From the Abbott Laboratories, North Chicago)

(Received for publication, April 21, 1948)

Experiments to determine the comparative nutritive value of partial acid hydrolysates of various proteins have been carried out for several years in this laboratory. Most of this work has involved studies of nitrogen balance in dogs wherein the hydrolysates were administered intravenously. Short term experiments, *i.e.* 4 to 5 days, were used to determine minimum nitrogen levels at which the N balance could be maintained (1, 2). In these experiments 2, 4, and 6 hour hydrolysates of casein and fibrin (3) all appeared to be well utilized and capable of maintaining the balance at levels of 120 to 150 mg. of N per kilo per day. The casein hydrolysates did not support the balance at this low level unless fortified with sulfur amino acids. In subsequent longer term experiments (4) fibrin hydrolysates proved adequate to support dogs in nitrogen balance at a level of 120 mg. of N per kilo per day for 15 weeks, whereas dogs on somewhat higher levels of casein hydrolysate failed completely in a few weeks. On the basis of these and other experiments it was concluded that short term nitrogen balance studies in normal adult dogs may be misleading and that long term experiments at low levels of nitrogen are more significant.

Another method of approach tested the ability of hydrolysates to bring about rapid recovery of severely depleted dogs when given in massive dosage (5). In these studies partial acid hydrolysates of fibrin were again better utilized than those of casein. Because the casein and fibrin hydrolysates did not appear to differ markedly in essential amino acid value (5), it was deduced that the better utilization of fibrin hydrolysate might be due to better utilization of the bound (peptide) amino acids of fibrin hydrolysate. Christensen, Lynch, Decker, and Powers (6) have reported a somewhat lower excretion of bound amino acids in humans following infusion of partial acid hydrolysates of fibrin than after infusion of an enzymatic digest of casein. These findings point to the need for intravenous studies in the final assessment of intravenous hydrolysates which contain a significant proportion of their total amino acids in bound form.

* Presented in part before the Division of Medicinal Chemistry of the American Chemical Society, New York, September 17, 1947.

Although there is an obvious advantage in the intravenous method, certain practical difficulties arise in the use of dogs as assay animals. Dogs available for use in most laboratories vary as to age, breed, and nutritional history and do not compare in degree of standardization as test agents with the rat. Also there are large quantitative differences between requirements for growth and maintenance. Hegsted, Hay, and Stare (7) have estimated that no more than 1.6 mg. of tryptophan and 15 mg. of isoleucine per kilo of body weight per day are required for nitrogen balance in the adult dog, whereas the similar requirements for maximum growth in rats were estimated to be 94 mg. and 880 mg. respectively. Furthermore the amino acid requirements of the rat are better known than are those of the dog. We were thus influenced to seek a rapid and fairly precise method for screening and control purposes with rats. A method which would clearly reveal the amino acid adequacy of essential amino acids in liquid protein hydrolysates, and would not necessitate drying the hydrolysates, appeared most desirable.

The method described herein is based on the rat repletion method of Wissler, Steffee, Woolridge, Penditt, and Cannon (8) and Frazier, Wissler, Steffee, Woolridge, and Cannon (9). These authors have established that lack of any one of nine amino acids essential for the adult rat results in prompt deficiency symptoms, which are as promptly corrected by return of the missing amino acid to the diet. Their results are qualitatively similar to those reported extensively by Rose (10). The repletion method is more rapid than the rat growth method and is particularly adapted to the problem in hand because of the ability of adult rats to consume large volumes of liquid nutrients, as previously described (8, 11). Several applications of the method are described.

EXPERIMENTAL

All rats used were from our own colony, which has been inbred for many years. Vigorous, rapidly growing, young adult male rats were raised on breeder stock diet until they weighed 160 to 220 gm. They were then placed on Diet NP4 for 12 days, during which they uniformly lost about one-fourth of their initial body weight. Shorter depletion periods did not give as satisfactory results as the 12 day depletion. The highly purified protein-free diet, Diet NP4, which contains only 0.02 to 0.05 per cent nitrogen, is made up as follows: sucrose 83, Salt Mixture 1 (U. S. P.) 4, agar 1.4, Primex 4.2, corn oil 4.2, cod liver oil 1.4, choline chloride 0.15, and inositol 0.14 gm.; thiamine hydrochloride 0.6, riboflavin 1.2, pyridoxine hydrochloride 0.6, calcium pantothenate 5.0, nicotinic acid 3.7, mixed tocopherols 2.5, and ascorbic acid 14 mg. per 100 gm. of diet.

The rats were supplied the 5 per cent hydrolysates in 60 cc. test-tubes

attached to the cages. Aluminum drinking fountains with rather broad planed rims were used. No loss of the solutions occurred from siphoning with this type of drinking tip. Unless otherwise indicated, no water was given. The non-protein diet was fed *ad libitum*. Final weighings were made 24 hours after the last hydrolysate feeding. In order to orient the rats equally the following procedure was adopted. After a 12 day depletion all rats are offered 40 to 50 cc. daily of a standard 5 per cent hydrolysate for 3 days. During this period the rats learn to drink at nearly the maximum rate. They are returned to the non-protein diet with water for 3 more days, during which they again lose weight. Rats thus prepared usually consume the entire allotment of hydrolysate subsequently offered to them, and continue to do so provided no amino acid deficiency is present. Groups of fifteen to thirty rats were prepared at a time. Groups for individual assays numbered four to seven rats. Because of the small size of the groups conclusions are based only on experiments which have been repeated two or three times with the same general results. In experiments in which the intake was limited to 0.12 to 0.32 gm. of N per rat per day, a single measured volume of solution was fed daily. In all instances in which there was good balance of the essential amino acids this volume was taken before bacterial growth became visible. During feeding *ad libitum* the drinking tubes were filled twice daily. An advantage of the method is the ease and accuracy of measuring the liquid and nitrogen intake each day.

The essential amino acids were determined in most of the hydrolysates studied. Tryptophan was determined colorimetrically directly on the unchanged hydrolysates by the method of Graham, Smith, Hier, and Klein (12). This modification of the method, which depends on the color formed by the reaction of tryptophan with *p*-dimethylaminobenzaldehyde, gives somewhat lower values for tryptophan in fibrin hydrolysates than does that of Horn and Jones (13), previously used in our laboratory. Cystine was determined by the method of Folin and Marenzi (14) and methionine as described by White and Koch (15). The remaining essential amino acids were determined microbiologically by the method of Stokes, Gunness, Dwyer, and Caswell (16). Free amino acid nitrogen determinations were made by the ninhydrin method of Van Slyke, Dillon, MacFadyen, and Hamilton (17).

Assay of Dried Proteins—For purposes of comparison a number of dried powdered proteins were fed in small cups attached to the cage wall by metal clips. Water was offered *ad libitum*. All proteins were fed at a level to supply 0.24 gm. of N per rat per day. The fibrin, casein, and wheat gluten supplements were completely, or nearly completely, consumed by all of the rats in these groups. The beef muscle, which had been extracted

with benzene, and the defatted whole egg were refused by some of the rats in these groups. The results are shown in Table I.

Repeated Use of Rats—The adaptability and economy of the method could be extended if rats could be used for repeated experiments. This procedure was tried through three regular runs of similar fibrin hydrolysates, starting with rats of original weights of 160 to 210 gm. In the second and third assays, we dispensed with the drinking trial because the rats were already trained to drink at the maximum rate. Results of the first two assays were quite similar.

Because the general condition of the rats appeared to deteriorate somewhat through three consecutive assays, further experiments were tried in

TABLE I

Assay of Dry Protein Supplements Fed Separate from Non-Protein Diet at Level of 0.24 Gm. of N per Day

Protein*	N content	No. of rats	Average intake of allotted N	Weight gain	
				Range	Average
	<i>per cent</i>		<i>per cent</i>	<i>gm.</i>	<i>gm.</i>
Fibrin.	15.1	4	100	58-60	58
Casein.	13.22	6	96	27-52	44.3
Wheat gluten. .	12.65	6	98	11-26	18.8
Beef muscle (defatted).	15.65	4	100	39-53	45.5
Whole egg (defatted)	12.16	3	91	52-57	54.7

* The casein, defatted beef muscle, defatted whole egg, and wheat gluten were kindly supplied by the Bureau of Biological Research of Rutgers University as part of a collaborative study. Two of six rats started on beef muscle and three of six rats on whole egg failed to take the supplements and are not included in the averages.

which the rats were returned to the stock breeder diet for 4 days prior to the third depletion. When this was done, the condition of the rats for the third assay was greatly improved, and responses very close to those of the prior assays were obtained. The variation in average weight gain in repeated assay of the same preparation in the same rats was less than 10 per cent. When minimum responses have been obtained on any assay, a transfer to the stock diet for 4 to 5 days has served well to prepare rats for further work.

In general the results reported herein involved only one use of the animals; however, the results at hand suggest that repeated use of the animals is feasible with proper conditioning between experiments.

Nitrogen Feeding Levels—Volumetric feeding of the liquid hydrolysates provided a good opportunity to study the response to different nitrogen levels. The observation was made that liquid feeding *ad libitum* could be used advantageously to reveal gross differences in amino acid adequacy of

hydrolysates, but that minor differences were sharpened by controlled isonitrogenous feeding. On *ad libitum* feeding, rats consumed as much as 80 cc. per day of certain hydrolysate solutions, equal to 0.5 gm. of N. The general intake, however, of well balanced hydrolysates was 55 to 60 cc. per day, equal to about 0.35 to 0.4 gm. of N. Ratios of nitrogen efficiency calculated from *ad libitum* feeding of different hydrolysates showed close correlation with weight gain. The present study was undertaken to determine the levels at which the nitrogen efficiency would be highest for a representative fibrin hydrolysate.¹ This level was then chosen for general assay purposes.

For the study herein reported we used a 5 per cent partial acid hydrolysate of fibrin with 5 per cent dextrose which contained about one-third of its amino acids in peptide form. The hydrolysate solids contained the following percentages of amino acids, calculated to 16 per cent N: isoleucine 5.1, leucine 7.5, valine 4.2, lysine 8.8, histidine 3.4, arginine 7.5, tryptophan 1.0, methionine 3.4, cystine 2.3, threonine 6.6, phenylalanine 3.4, tyrosine 1.5, glutamic acid 13.9. Four groups of rats were oriented to liquid feeding in the usual way and were fed the liquid hydrolysate at levels of 0.12, 0.18, 0.24, and 0.3 gm. of N per rat per day. The amounts of liquid hydrolysate fed daily to the rats in each group were 17.2, 25.8, 34.4, and 43 cc., respectively. All rats were allowed water and the non-protein diet *ad libitum*. Very little water was taken, whereas the hydrolysate allotments were consumed by all rats throughout. The rats on the lowest level generally drank their daily allotment within an hour after it was offered.

The results are shown in Fig. 1. The nitrogen efficiency ratios on the four groups were 12.1, 12.5, 10, and 9.3, respectively.

Time of Assay—Although a 12 day recovery period appears advantageous for most assays, extension of the time to 15 to 18 days may be needed to develop significant differences between materials of rather close nutritive value. For instance, partial acid hydrolysates made from fibrin which contained a considerable amount of plasma protein appeared to contain a limiting level of isoleucine, *i.e.* about 3.0 per cent on a dry basis. In order to determine the effect of added isoleucine, two equal groups of four rats each were formed. After a 12 day depletion the separate groups were fed *ad libitum* the 5 per cent hydrolysate and the hydrolysate to which was added 0.2 per cent of a leucine-isoleucine concentrate. This addition was equal to about 1 per cent of each amino acid on a dry basis. The isoleucine concentrate contained 43 per cent isoleucine and 45 per cent leucine as determined by microbiological assay.

¹ Partial acid hydrolysate of purified fibrin for intravenous administration is sold under the trade name, Aminosol.

The results are shown in Fig. 2 together with a record of the 12 day weight recovery of six rats on a hydrolysate of purified fibrin which contained 4.6 per cent isoleucine. All of the rats which received the extra isoleucine had gained more than any of the rats on the unfortified hydrolysate after the 18th day of the assay and the difference appeared significant. Repetition of this experiment in a 12 day assay again revealed a small average increase in growth response on addition of synthetic DL-isoleucine to the crude fibrin hydrolysate. These results indicate that a longer

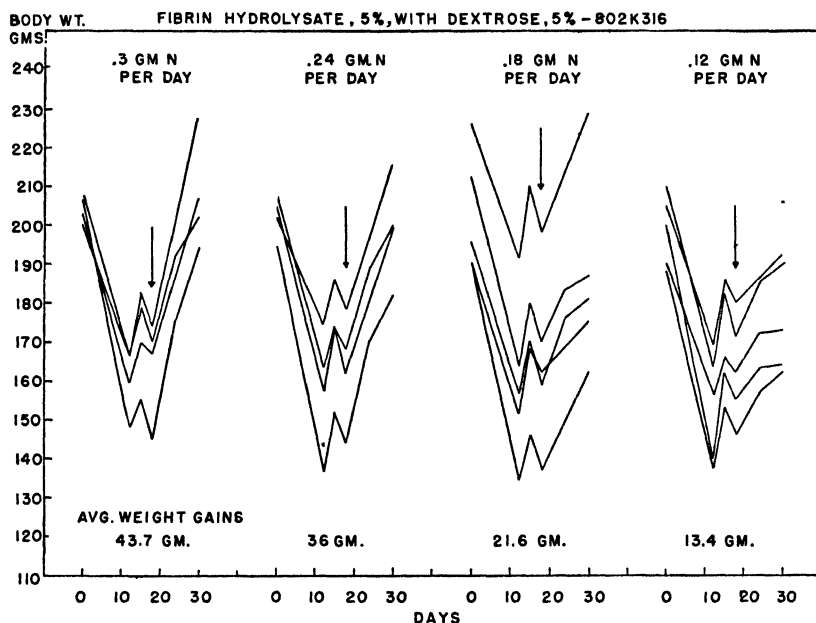


FIG. 1. The arrows indicate the point at which feeding the fibrin hydrolysate at the four different intake levels was started. All rats were prepared for assay by a 12 day depletion period, a 3 day drinking trial on a standard fibrin hydrolysate, and a 3 day redepletion.

assay period than 12 days may be desirable, depending on the purpose for which it is used. For purposes of routine testing, a somewhat shorter period than 12 days may prove adequate, particularly when the nitrogen intake is limited to a critical level.

The isoleucine requirement for a maximum rate of repletion was calculated to be somewhat in excess of 75 mg. per rat per day.

Requirement for Tryptophan—Frazier *et al.* (9) indicated that in complete amino acid mixtures an intake of 18 mg. per day of DL-tryptophan supplied the need for this amino acid for good weight recovery in adult protein-depleted rats, but that 9 mg. of DL-tryptophan were distinctly limiting and

gave only a very small response. As a result of many experiments the fact became clear that partial acid hydrolysates of fibrin which contained upwards of 0.9 per cent natural tryptophan on a dry basis gave uniformly rapid weight recovery in depleted rats. When fed *ad libitum*, such hydrolysates supplied about 24 mg. of tryptophan per rat per day, with a 12 day weight recovery of about 60 to 65 gm. Hydrolysates which contained 0.6 per cent tryptophan under similar conditions supported an

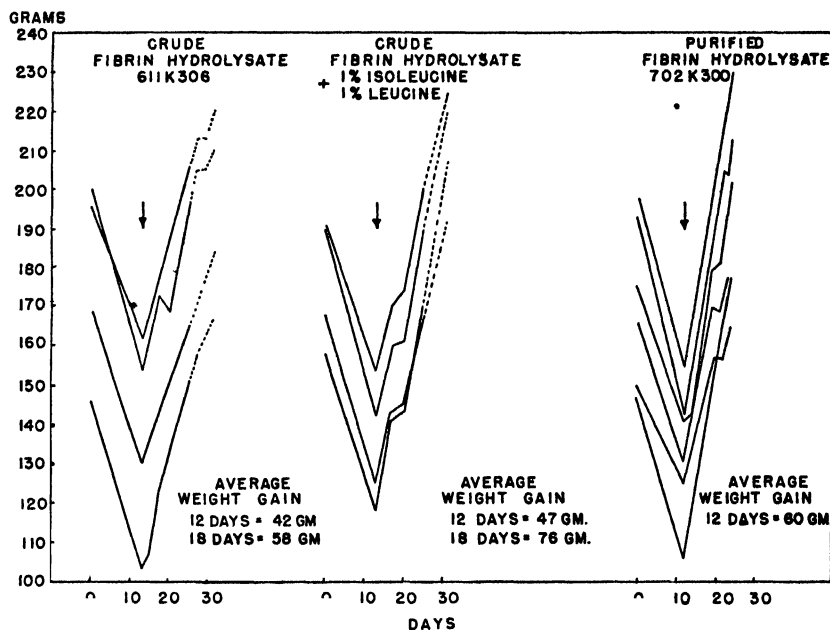


FIG. 2. Weight response at 12 and 18 days of protein-depleted rats (males) to *ad libitum* feeding of partial acid hydrolysates of crude fibrin alone and fortified with equal parts of leucine and isoleucine at a level of 1 per cent of the hydrolysate solids. Comparison with partial acid hydrolysate of purified fibrin. The solutions each contained 5 per cent hydrolysate and 5 per cent dextrose. The solid lines indicate the first 12 days of the supplement; the broken lines, the 6 additional days of supplement.

average weight gain of only about 40 gm., with a tryptophan intake of about 14 mg. per rat per day.

In order to make the effect of a limiting deficiency of tryptophan more critical, experiments were run in which the hydrolysate intake was limited to supply 0.24 gm. of N per rat day. Previous work had shown that the maximum weight recovery on various hydrolysates fed at this level is 50 to 55 gm. in 12 days. For the experiments a 5 per cent fibrin hydrolysate was selected which was known to contain a limiting level of tryptophan; *i.e.*, 0.68 per cent on the basis of the hydrolysate solids. To part of this

was added an amount of DL-tryptophan to give 0.92 per cent on a dry basis. The experiment was carried out with two groups of six rats each, as shown in Fig. 3.

The considerable difference in weight gain for the two groups of rats is shown in Fig. 3. The 12 mg. daily level of tryptophan is clearly too low for best performance. The level of 16.5 mg. is thought to be about optimum at the level of nitrogen intake studied. A somewhat higher level,

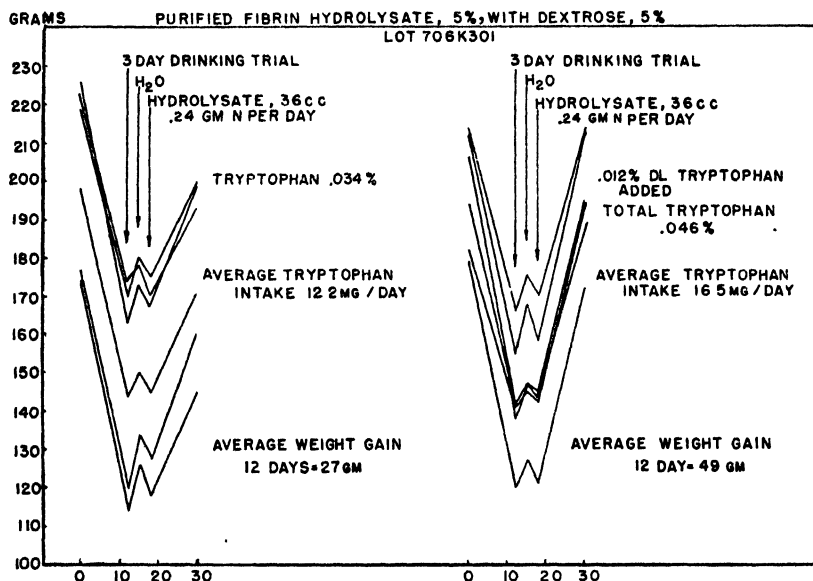


FIG. 3. Weight response of protein-depleted rats (males) to controlled feeding of a partial acid hydrolysate of purified fibrin alone and with 0.24 per cent DL-tryptophan added on the hydrolysate solids. The solutions fed contained 0.24 gm. of N per day. The assay includes a 3 day orientation period during which the liquid hydrolysate was fed *ad libitum*, followed by a 3 day depletion before the assay.

i.e. 18 to 20 mg. per rat per day, is required for the maximum response of about 60 to 65 gm. under conditions of *ad libitum* feeding.

Effect of Degree of Hydrolysis—The availability of partial acid hydrolysates of fibrin of varying degrees of hydrolysis offered the opportunity to study the effect of the progressive destruction of streptogenin on nutritive value. Five separate hydrolysates varying widely in the degree of hydrolysis were compared in the repletion test. The hydrolysis conditions used were similar in each instance except that the degree of hydrolysis was varied by the time of heating in acid. The ratio of free amino acid nitrogen to total nitrogen ranged from 42 to 74 per cent in the hydrolysates studied. Complete hydrolysates of fibrin have a ratio of 75 to 76 per cent.

The tryptophan was completely destroyed in Lot 705K308, in which the hydrolysis was nearly complete. When necessary, L-tryptophan was added so that the content of all hydrolysates would be similar, *i.e.*, 1.0 per cent tryptophan on the basis of 16 per cent nitrogen. Also all hydrolysates were similarly fortified to supply 3.2 per cent methionine and 2.7 per cent cystine.

The strepogenin content of the hydrolysates was determined microbiologically with *Lactobacillus casei*, as described by Sprince and Woolley (18). According to their practice Wilson liver fraction L was arbitrarily

TABLE II

Effect of Degree of Hydrolysis and Strepogenin Content of Acid Hydrolysates of Fibrin on Repletion Response to Feeding ad Libitum

Fibrin hydrolysate,* lot No.	Hydrolysis† hrs	COOH-N to total N per cent	12 day repletion response			Strepogenin content (<i>Lactobacillus</i> <i>casei</i> assay)‡
			No. of rats	Range gm.	Average gm.	
702K300	6	42	9	47-74	60	2.3
704K300	7	44	9	40-56	51	1.6
702K302	8	48	6	47-58	55	1.3
706K312	11	55	6	47-65	56	0.7
705K308	18	74	7	44-80	63	0
Complete hydroly- sate		75-76				0

* All lots contained 5 per cent fibrin hydrolysate and 5 per cent dextrose, and were made to contain equal levels of tryptophan, methionine, and cystine.

† Hydrolysis was carried out at 10 to 20 per cent protein concentration with a ratio of H_2SO_4 to fibrin of 1.2:1. The complete hydrolysate was further hydrolyzed with 8 N HCl for 8 hours.

‡ Liver fraction L (Wilson) = 1.

assigned a value of unity and the potency of the hydrolysate solids was expressed in relation to this standard.

The five hydrolysates made to 5 per cent solids were assayed by the rat repletion method with feeding *ad libitum*. From six to eleven rats were used in each group. Records of liquid intake were kept throughout the 12 day period so that nitrogen efficiency ratios could be calculated. All of the hydrolysates were well taken, the intake averaging about 55 to 60 cc. per day.

The degree of hydrolysis, the average 12 day weight gains, and the relative strepogenin content of the five hydrolysates are shown in Table II. The data indicate that there is no failure in nutritive value as measured by this test following destruction of almost all of the strepogenin originally present in the protein. Nitrogen efficiency values in the narrow range of 12.6 to 13.8 were obtained for the different hydrolysates.

The lot of fibrin hydrolysate in which hydrolysis was nearly complete, Lot 705K308, was also tested at the level of 0.24 gm. of N. The average weight gain of six rats under these conditions was 52 gm. (range, 41 to 60 gm.), which is about the maximum obtained with partial hydrolysates of fibrin.

Partial Acid Hydrolysates of Casein—Experiments with partial acid hydrolysates of casein are particularly interesting with regard to the

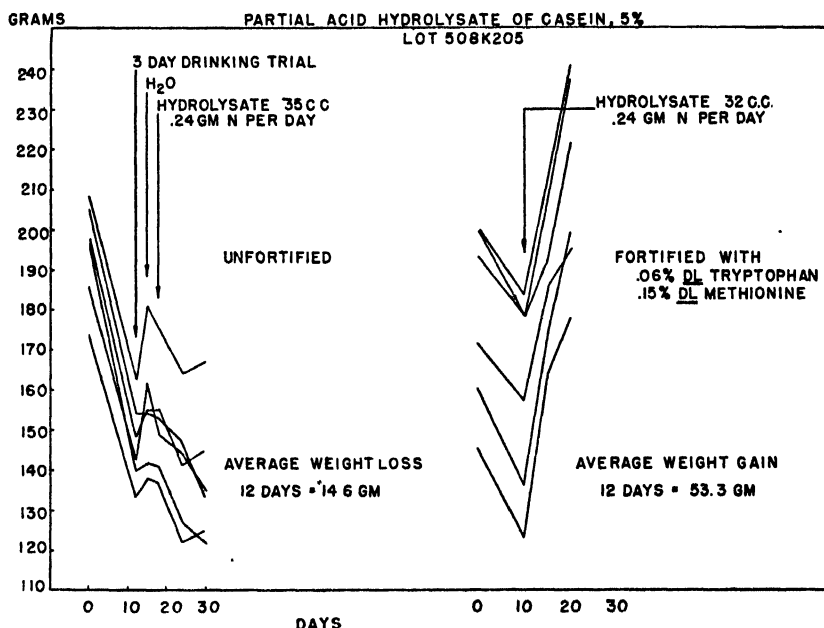


FIG. 4. Weight response of protein-depleted rats (males) to the controlled feeding of partial acid hydrolysates of casein alone and fortified with DL-tryptophan and DL-methionine. New rats after a 3 day orientation period were used in the first instance; rats twice depleted in the latter case. The solutions contained 5 per cent hydrolysate only.

utilization of the sulfur amino acids. Experiments were carried out with a casein hydrolysate which contained about 75 per cent bound amino acids. The essential amino acid content, calculated to 16 per cent N, is as follows: isoleucine 4.5, leucine 8.4, valine 6.4, threonine 4.2, methionine 2.8, cystine 1.5, phenylalanine 3.7, tyrosine 4.2, tryptophan 0.4, lysine 8.1, histidine 3.2, and arginine 3.4. The hydrolysate was fortified during manufacture with 0.8 per cent cystine above the normal level of 0.7 per cent. Assay results on this hydrolysate at a level of 0.24 gm. of N per day are shown in

Fig. 4. Only 47 per cent of the liquid allotment was taken and a weight loss occurred in all rats.

The tryptophan deficiency was corrected by addition of DL-tryptophan to a level of 1.6 per cent on 16 per cent N. DL-Methionine was added to supply a total of 6.29 per cent. The hydrolysate was fed at a level of 0.24 gm. of N per day and the average intake was 88 per cent of the allotment. Growth responses for the hydrolysate fortified in this way are shown in Fig. 4.

Further experiments were conducted in which the original hydrolysate was fortified with DL-tryptophan at the same level as above. The casein hydrolysate fortified with tryptophan supported an average weight recovery of 30 gm. (range 20 to 41 gm.) in 12 days in six rats. The rats took 93 per cent of the allotment of 0.24 gm. of N per rat per day.

DISCUSSION

The primary rôle played by the "indispensable" amino acids, lysine, histidine, isoleucine, leucine, valine, threonine, tryptophan, and phenylalanine, has been clarified by Rose and his coworkers for growth in rats and maintenance in adult humans (10), and by Cannon's group (9) for repletion in adult protein-depleted rats. The present method imposes highly critical conditions in the use of still growing, nearly adult rats for depletion. The stimuli for growth and repletion occur simultaneously and deficiencies in assay materials promptly become manifest.

Frazier *et al.* (9) have reported that amino acid mixtures patterned after casein support as great or greater repletion responses as an isonitrogenous amount of casein. Our experience (11) has been that amino acid mixtures produce a somewhat greater response than casein. This has been true, despite the unavailability of the D forms of valine, isoleucine, and threonine used in the mixtures. Direct comparisons are further complicated by the fact that casein contains about 10 per cent of its nitrogen in the amide groups of asparagine and glutamine. The finding that progressive destruction of streptogenin does not reduce the repletion response to *ad libitum* feeding strongly supports the thesis that adult protein-depleted rats do not require streptogenin for maximum recovery.

Somewhat opposed to the hypothesis that proteins and protein hydrolysates contain amino acids combined in a way which provides a nutritional advantage, such as that ascribed to streptogenin for the growth of young rats (19), are the repeated findings in this laboratory that complete acid hydrolysates of proteins fortified with tryptophan are not generally inferior, and may even be superior, to the original proteins. Risser (20) reported that complete hydrolysates of casein fortified with tryptophan

and cysteine are slightly more effective in maintaining nitrogen balance orally in dogs at minimum levels than is whole casein fortified with the same amount of cysteine. As previously discussed, partial acid hydrolysates of casein appear to be less well utilized on injection than partial acid hydrolysates of fibrin (4, 5). In the present experiments there is further evidence that the failure in utilization of casein and casein hydrolysates involves the sulfur amino acids, particularly methionine.

The following average weight gains for 12 days, from the above data and Table II, are illustrative: casein 44 gm., casein hydrolysate fortified with tryptophan and cysteine 30 gm., casein hydrolysate fortified with tryptophan, cysteine, and methionine 53 gm., and fibrin 58 gm. The total sulfur amino acid content of these preparations was determined as 3.6, 4.3, 7.8, and 3.8 per cent respectively. The methionine contents of casein and fibrin were found by analysis, and are generally reported to be about 3 and 2.2 per cent respectively. In these experiments, as in the previous work with dogs (5), fortification of partial acid hydrolysates of casein with cysteine to a level of total sulfur amino acids in excess of that in fibrin is insufficient to correct the sulfur amino acid deficiency. Fortification with adequate methionine, however, appears to improve the nutritive character of this type of casein hydrolysate greatly. It is of further interest to note that pure amino acid mixtures, patterned after casein and made to contain about 3.8 per cent total sulfur amino acids, supported nearly maximum repletion responses and no evidence of a sulfur amino acid deficit was apparent (11). Experiments are under way to determine the minimum sulfur amino acid requirements for repletion. This appears from the data in hand to be no more than 70 mg. per rat per day.

The ratio of sulfuric acid to fibrin used in these experiments was 1.2:1, about 50 per cent greater than the ratio used previously (3-5). Experience in this laboratory with the hydrolysis of proteins, particularly recent work by Dr. G. F. Lambert, has indicated that fibrin is unusual with regard to the ease with which it undergoes acid hydrolysis. Although complete destruction of tryptophan, as well as streptogenin, occurred under the conditions used for complete hydrolysis, no significant destruction of other essential amino acids was thought to occur. Casein is considerably more resistant to acid hydrolysis than fibrin and requires rather drastic conditions for completion of hydrolysis. Because of the many interactions which take place during the hydrolysis of proteins it is difficult to assess the resulting hydrolysates in relation to the whole protein. The use of fibrin may reduce these uncertainties to some extent; however, the final answer must be based on comparisons between purified proteins and their counterpart mixtures of pure L-amino acids.

SUMMARY

A relatively rapid method for assaying the nutritive value of liquid protein hydrolysates, based on weight regeneration in protein-depleted rats, is described. A feeding level of 0.24 gm. of N per rat per day was found to give the highest nitrogen efficiency ratio in the case of a partial acid hydrolysate of fibrin, and was adopted as a standard feeding level. Several whole proteins were assayed as dry supplements separate from the diet at the 0.24 gm. of N level.

Partial acid hydrolysates of fibrin were assayed with regard to the optimum level of tryptophan and isoleucine. The requirement for tryptophan for maximum weight gain was estimated at about 18 to 20 mg. per day. The similar requirement for isoleucine was somewhat greater than 75 mg. per day. Experiments with partial acid hydrolysates of casein indicated that the sulfur amino acids contained therein are not completely utilized when taken orally by the rat.

The degree of hydrolysis of partial acid hydrolysates of fibrin did not appear to alter the repletion responses up to the hydrolysis point at which 97 per cent of the amino acids were in free form and no streptogenin remained.

Thanks are expressed to P. N. E. Naidu, in charge of Aminosol manufacture, for supplies of many of the materials studied, to Elsa Proehl for COOH-N determinations, to Eleanor Willerton for microbiological amino acid analyses, and to E. O. Krueger for chemical analyses of amino acids.

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THE RÔLE OF BIOTIN AND ADENYLIC ACID IN AMINO ACID DEAMINASES

By HERMAN C. LICHSTEIN AND JOHN F. CHRISTMAN

(From the Department of Bacteriology, University of Tennessee, Knoxville)

(Received for publication, May 3, 1948)

Within the past year the mode of action of biotin has become increasingly clarified. The relation of this vitamin to oxalacetic acid decarboxylation was discovered independently in at least four laboratories (1-4), while its apparent rôle in the synthesis of oleic acid has been suggested by Williams *et al.* (5) and Snell *et al.* (6). Biotin has further been shown to activate the deamination of aspartic acid, serine, and threonine (7). This paper is concerned with biotin activation of the deaminases.

Methods

The organisms employed in these studies were *Escherichia coli* (Gratia), *Escherichia coli* (10B3), *Proteus vulgaris*, *Aerobacter aerogenes*, and *Bacterium calaveris* (Gale). They were grown for approximately 16 hours at 27-30° in a medium composed of 1 per cent each of tryptone and yeast extract and 0.5 per cent K_2HPO_4 ; the final pH was 6.9 to 7.2. In certain instances this medium was supplemented with 0.1 per cent formate. Cells were harvested by centrifugation, washed once with distilled water, and suspended in M phosphate, pH 4, to give about 1 mg. of bacterial nitrogen per ml. Biotin deficiency was obtained as previously described (3, 7) by holding the cell suspensions at pH 4 in phosphate buffer for 30 to 60 minutes at 20-30°. The deamination experiments were performed at either pH 4 or 7 in phosphate buffer at 37°. After incubation in the presence of an amino acid substrate the reaction was stopped with 100 per cent trichloroacetic acid, and ammonia was determined colorimetrically (Klett-Sumerson photoelectric colorimeter) on aliquots of the centrifuged samples. The biotin employed was the free form¹ and the adenylic acid was the adenosine-5-phosphoric acid.² Further details are included with the data.

Results

The data given in Table I demonstrate that biotin stimulates aspartic, serine, and threonine deaminases. Biotin alone fully replaces the mixture

¹ We are grateful to Merck and Company, Inc., Rahway, New Jersey, for supplies of this material.

² Kindly supplied to us by the Ernst Bischoff Company, Ivoryton, Connecticut.

of all the known members of the vitamin B complex, while such a mixture without biotin gives little or no stimulation.

TABLE I

Biotin Activation of Aspartic Acid, Serine, and Threonine Deaminase

Cells grown as described in the text, aged at pH 4 in M phosphate at 25–30°. Reaction run at pH 7, 37°, 20 to 30 minutes; volume 2 ml. Amino acids added at 0.005 M final concentration. The increase in ammonia over samples stopped at zero time was taken as an index of deamination.

Organism	Amino acid	Ammonia nitrogen produced			
		No additions	Vitamins less biotin*	Vitamins†	Biotin, 0.1 γ per ml.
<i>E. coli</i> (Gratia)	L-Aspartic	γ	γ	γ	γ
		8.1	7.7	10.1	10.0
		8.3	9.2	28.4	25.8
		11.0	8.8	23.5	26.0
		9.3	10.6	24.5	23.9
		6.6	8.4	23.5	21.6
	DL-Serine	10.9	11.2	46.5	40.1
		23.0	23.0	31.9	30.5
		21.6			31.3
		25.1	25.2		46.4
		8.8			15.7
<i>P. vulgaris</i>	L-Aspartic	1.5			14.8
		5.9	6.5		20.3
		3.9			6.8
	DL-Serine	5.3			10.4
		14.6	14.4		27.2
<i>E. coli</i> (10B3)	L-Aspartic	5.4			10.4
	DL-Threonine	7.8			14.9
		6.2	6.5	12.8	12.9
<i>B. cadaveris</i>	L-Aspartic	3.6			8.9
		4.1	4.5		12.7
		0			7
	DL-Serine	2.9			7
		6.3	6.3	12.9	13.3
	DL-Threonine	2.2			5.8
		2.4	3.0	8.9	8.6

* Vitamins added per ml., nicotinic acid 2.5 γ, *p*-aminobenzoic acid 1 γ, riboflavin 0.5 γ, pantothenic acid 0.5 γ, thiamine 1 γ, folic acid 0.5 γ, pyridoxal 5 γ.

† As vitamins less biotin with free biotin added to yield 0.1 γ per ml. of the reaction mixture.

Attempts to reverse deamination of serine and threonine have so far been unsuccessful, while with aspartic acid such reversal is readily demonstrable. Using NH₄Cl and malic acid, we have been able to follow the

disappearance of ammonia and to show that biotin and adenylic acid are also involved in this reaction. Representative data are given in Fig. 1. Results with fumarate have been variable, probably because of permeability effects. We have previously shown (3) that the product of aspartic acid deamination in these cells is fumaric acid and not malate, but that malate and fumarate exist in equilibrium because of an active fumarase present in these cells.

Biotin fails to stimulate the deamination of alanine, phenylalanine, and

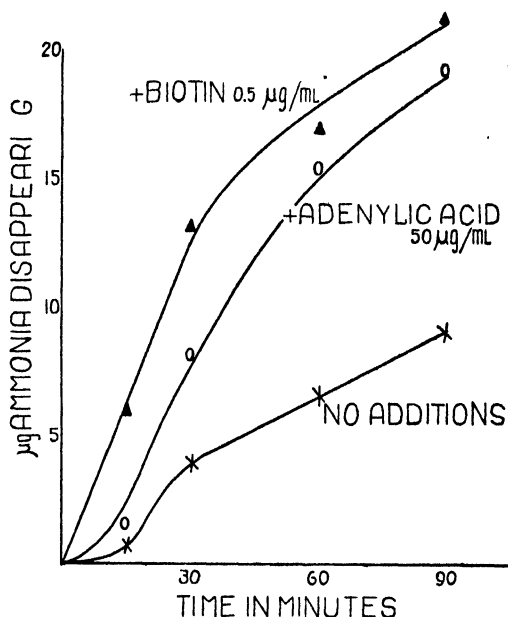


FIG. 1. Reversibility of aspartic acid deaminase in *Bacterium cadaveris*. Cells aged at pH 4 in M phosphate at 20° for 60 minutes. Reaction run at pH 7, 37°. 0.03 M malic acid + 20 γ of ammonia as NH₄Cl. Reaction volume 2 ml.

methionine (chosen as typical of the substrates of the D- or L-amino acid oxidases) and glutamic acid in comparable experiments in which it was effective in stimulating aspartic deaminase (Table II). The glutamic deaminase in *Aerobacter aerogenes* has given variable results, as shown by the data in Table II. In one of five experiments performed, a definite stimulation with biotin was recorded. However, to determine whether this is a direct biotin effect on glutamic deaminase or an indirect action due to transamination of glutamic acid to aspartic acid (with oxalacetic acid present in the cell) and subsequent deamination of the latter amino acid requires further study. It may be noted that glutamate deamination is a

somewhat different process than aspartate deamination in that the former is reported to involve a diphosphopyridine nucleotide- or a triphosphopyridine nucleotide linked dehydrogenation with the formation of the keto acid (8).

Gale (9, 10), working with aspartic and serine deaminases in *Escherichia*

TABLE II

Failure of Biotin to Stimulate Deamination of Certain Amino Acids

Conditions as for Table I.

Organism	Amino acid	Ammonia nitrogen		Organism	Amino acid	Ammonia nitrogen	
		No ad-ditions	Biotin, 0.05 γ per ml.			No ad-ditions	Biotin, 0.05 γ per ml.
<i>E. coli</i> (86G)	L-Aspartic	5.8	17.7	<i>A. aerogenes</i>	L-Aspartic	6.1	10.7
	DL-Alanine	10.2	9.4		DL-Alanine	3.2	3.4
	DL-Phenylalanine	4.8	4.6		DL-Methionine	3.7	3.7
					L-Glutamic	3.2	3.1
	L-Aspartic	10.3	30.3				
	L-Glutamic	4.2	4.0		L-Aspartic	4.6	12.3
	DL-Alanine	6.1	7.1		DL-Alanine	4.5	4.4
	DL-Methionine	6.6	6.4		DL-Methionine	4.0	4.2
	L-Aspartic	6.8	21.8		L-Aspartic	33.0	43.1
	L-Glutamic	3.9	3.9		L-Glutamic	11.1	19.1
	DL-Alanine	7.0	7.1				
	DL-Methionine	4.9	4.2		L-Aspartic	6.1	10.7
	DL-Phenylalanine	4.8	4.4		L-Glutamic	2.7	3.2
<i>E. coli</i> (10B3)	L-Aspartic	5.4	10.4		DL-Methionine	3.1	2.7
	L-Glutamic	4.0	4.2				
	DL-Alanine	4.4	4.1		L-Aspartic	5.9	15.9
	DL-Methionine	3.2	2.9		L-Glutamic	6.9	7.0
	DL-Phenylalanine	2.7	2.8				
					L-Aspartic	6.4	10.6
					L-Glutamic	4.8	5.0

coli, reported that washed suspensions of these cells lost activity on standing and that the activity could be recovered by adding boiled cell suspensions or other materials. Further work showed that the killed cell substance could be replaced by adenylic acid and its breakdown products, the most active of which was adenosine. However, this was only true for aspartic deaminase, since adenylic acid in very low concentrations prevented both loss of activity and recovery in the case of serine deaminase. It was there-

fore of interest to determine whether adenylic acid stimulated the deficient cells obtained by our aging technique, which respond to biotin. The results of such experiments (Table III) show that muscle adenylic acid can replace biotin in aspartic, serine, and threonine deaminase, and that no additive effect is obtained by combining adenylic acid and biotin. Although the data presented here with aspartic deaminase are in general agreement with those of Gale (9), our results with serine deaminases are distinctly at variance with Gale's work (10).

TABLE III

Adenylic Acid Stimulation of Aspartic Acid, Serine, and Threonine Deaminase
Conditions as for Table I.

Organism	Amino acid	Ammonia nitrogen			
		No additions	Biotin, 0.5 γ per ml.	Adenylic acid 50 γ per ml.	Adenylic acid + biotin
<i>E. coli</i> (Gratia)	L-Aspartic	7	7	7	7
		8.3	25.5	23.8	23.1
		11.0	25.0	27.0	23.6
		8.1	10.0	10.6	10.9
<i>B. cadaveris</i>	DL-Serine	4.4	6.9	5.5	
		6.3	12.1	11.6	11.9
	L-Aspartic	0	5.7	5.3	
		0	7.0	12.1	
	DL-Serine	12.6	12.6	20.9	
		6.5	10.9	10.8	
	L-Aspartic	6.8	9.2	10.1	
	DL-Threonine	4.4	8.8	8.8	
	L-Aspartic	6.6	9.0	8.6	
	DL-Serine	4.8	7.3	7.0	
<i>P. vulgaris</i>	DL-Threonine	4.2	7.0	6.2	
	L-Aspartic	3.9	6.8	6.8	
	DL-Serine	7.7	12.1	11.5	

Further experiments were designed to determine the relative levels of each substance required to stimulate aspartic deaminase in *Bacterium cadaveris*. The data (Table IV) show a striking difference in the concentration of biotin and of adenylic acid required to activate this enzyme. In Experiment 1 the aging process resulted in cells which were completely unable to deaminate aspartic acid without additions. The biotin levels tested were 0.001 to 5 γ per 2 ml. of reaction volume. All levels were effective and no end-point was reached. On the other hand, 1 γ of adenylic acid seemed to be necessary for good stimulation and 0.1 γ gave very little activity. In Experiment 2 the dilutions of both substances were carried

further in an attempt to get a good end-point. The aging process was not as effective as in Experiment 1, but significant stimulations were obtained.

TABLE IV

Relative Concentration of Adenylic Acid and Biotin Required to Stimulate Aspartic Deaminase in Bacterium cadaveris

Conditions as for Table I.

Experiment No.	Biotin per 2 ml.	NH ₃ production	Adenylic acid per 2 ml.	NH ₃ production
	γ	γ	γ	γ
1	0	0	0	0
	5	12.4	100	12.1
	1	7.5	10	12.1
	10^{-1}	7.0	1	7.3
	10^{-2}	7.0	0.1	2.0
	10^{-3}	7.0		
2	0	5.9	0	5.9
	1	10.2	100	10.4
	10^{-1}	8.8	10	10.6
	10^{-2}	8.8	1	6.5
	10^{-3}	9.7	0.1	6.5
	10^{-4}	11.0	0.01	6.6
	10^{-5}	11.0		
	10^{-6}	6.5		
	0	2.7	0	2.7
3	10	7.5	10	11.2
	1	13.5	1	11.2
	10^{-1}	7.5	0.1	6.8
	10^{-2}	14.6	0.01	2.7
	10^{-3}	7.5		
	10^{-4}	7.1		
	10^{-5}	7.5		
	10^{-6}	7.5		
	10^{-7}	3.0		
4	0	5.7	0	5.7
	5	13.8	100	14.9
	1	11.6	10	14.5
	10^{-1}	12.3	1	9.4
	10^{-2}	13.2	0.1	9.2
	10^{-3}	13.2	0.01	6.7
	10^{-4}	13.0	0.001	6.6
	10^{-5}	10.8		
	10^{-6}	8.8		
	10^{-7}	5.9		
	10^{-8}	6.4		

The data obtained show that 10^{-5} γ of biotin is sufficient to stimulate the aged cells fully, whereas the activity of 10^{-6} γ is considerably less. The

end-point for adenylic acid is somewhere between 1 and 10 γ ; so that biotin is roughly 100,000 times more effective than adenylic acid. In Experiment 3 the aging process was very effective, resulting in cells showing very limited activity. Significant stimulations were recorded for biotin through 10^{-6} γ per 2 ml., while 0.1 γ of adenylic acid was required for similar stimulation. In Experiment 3 biotin was about 10,000 times more effective than adenylic acid. The deficiency obtained in Experiment 4 was almost identical with that in Experiment 2. Significant biotin stimulations were obtained through 10^{-6} γ per 2 ml., while adenylic acid was required in a concentration of 0.1 γ per 2 ml. to give equal stimulation, again making biotin about 10,000 times more effective than adenylic acid (see Fig. 2).

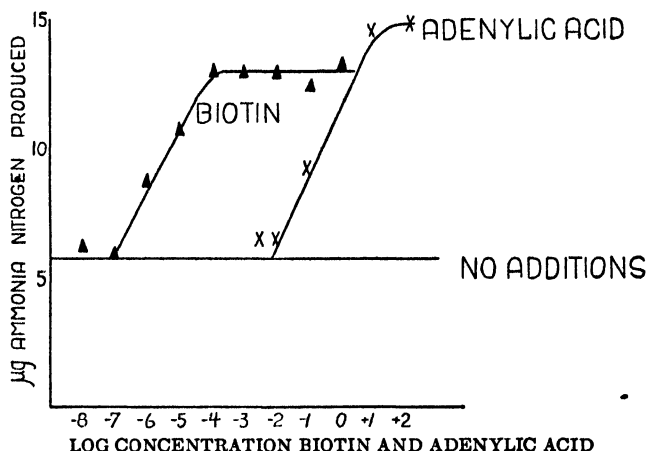


FIG. 2. Relative concentrations of adenylic acid and biotin required to stimulate aspartic acid deaminase in *Bacterium cadaveris*.

The logical question arises as to the rôle of adenylic acid in the deamination of aspartic acid, serine, and threonine. Although on the basis of the data cited (Table IV) adenylic acid is roughly 10,000 times less effective in stimulating the deficient cells than is biotin, it does nevertheless fully stimulate the aged cell. Controlled experiments show that under the conditions of the experiments there is no ammonia released from adenylic acid itself; so that increases in ammonia are due to deamination of added amino acid.

Several hypotheses were considered in an attempt to clarify the rôle of biotin and adenylic acid in the activation of these amino acid deaminases. The first of these was that the adenylic acid employed in these studies contained biotin as a contaminant. Such a hypothesis could be tested experimentally by microbiological assay for biotin. The methods em-

ployed were those given by Snell *et al.* (11) with *Saccharomyces cerevisiae*. The results obtained from such assays revealed that 1 γ of adenylic acid was equivalent to 10^{-10} γ of biotin on the basis of stimulation of the growth of *Saccharomyces cerevisiae* in a chemically defined medium lacking only in biotin. Since to account for the activation of the deaminases the adenylic acid should have contained 10^{-4} γ of biotin per microgram, it is unlikely that adenylic acid stimulation of aspartic, serine, and threonine deaminases is due to its biotin content.

Hypotheses other than biotin contamination of adenylic acid were developed; namely, (1) biotin may not be the coenzyme of these deaminases but functions in some manner in the production of adenylic acid, (2) adenylic acid may function as a non-specific energy source supplying the energy necessary to synthesize the active coenzyme form of biotin, (3) adenylic acid may be specifically necessary to phosphorylate biotin, (4) adenylic acid may combine with biotin to form a coenzyme similar in structure to diphosphopyridine or triphosphopyridine nucleotide. Attempts were made to put these hypotheses to experimental test.

It was felt that time curves would shed light on the first hypothesis, since, if biotin activation of these deaminases is due to an indirect effect, namely its necessity in the formation of adenylic acid, then there should be a definite lag period before biotin stimulation is noted and essentially none with adenylic acid. The data (Table V) presented are for six typical experiments. In all cases biotin stimulation was noted before adenylic acid stimulation, and in all but one instance biotin activation was present at the first time interval. These data suggest that the first hypothesis is unsound and that biotin must be directly associated with the aspartic acid deaminase. It must be pointed out that the lag in adenylic acid stimulation could well be due to permeability factors in the living cell. It should be noted that in three of the six experiments cited, in which the time curves were extended to 60 minutes, adenylic acid stimulation of aspartate deaminase at pH 7 exceeded that produced by biotin. This was taken as an indication that adenylic acid may be serving as an energy source either specifically or non-specifically (hypotheses (2) and (3)) and that the aged cells lose the ability to synthesize the coenzyme form of biotin in the absence of a suitable energy supply. Similar results have been obtained for serine and threonine deaminases. Further experiments were designed to study biotin and adenylic acid stimulation at pH 7 and 4 since the latter pH should be low enough to reduce markedly or even inhibit entirely the synthetic mechanisms of the cell. It was also considered advisable to include the breakdown products of adenylic acid, namely adenine and ribose, and to check the specificity by using guanine and xy-

Results of three typical experiments are given in Table VI and the data of one are graphically presented in Fig. 3.

It may be seen that the activation of aspartic acid deaminase by all substances tested with the exception of biotin differs at pH 7 and 4. Stimulation by adenine, guanine, xylose, and ribose, while noted at pH 7, is not present at pH 4, suggesting that these substances may stimulate only under

TABLE V

Time Required for Biotin and Adenylic Acid Stimulation of Aspartic Acid Deamination in Bacterium cadaveris

Conditions as for Table I.

The figures represent the micrograms of ammonia nitrogen produced after subtracting the micrograms of ammonia present in similar cell suspensions incubated at the times given without added aspartate.

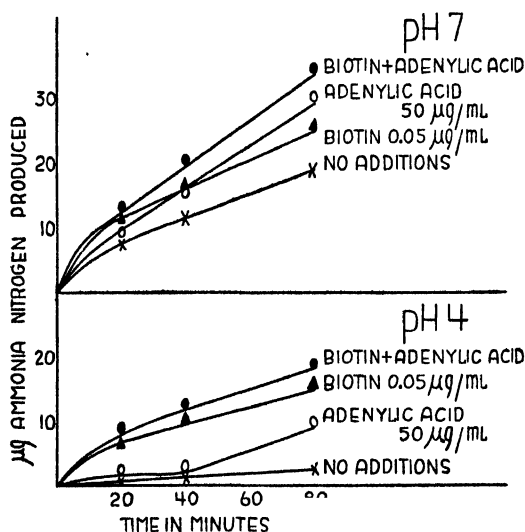
Additions	1 min.	5 min.	10 min.	15 min.	20 min.	30 min.	60 min.
None.....	0	2.2	4.2		6.2		
0.0005 γ biotin.....	2.2	4.6	7.3		9.3		
50 γ adenylic acid.....	0.2	2.6	3.7		6.4		
None.....	0	1.3	5.1		5.1		
0.0005 γ biotin.....	3.5	4.6	7.7		8.4		
50 γ adenylic acid.....	0.4	1.1	8.4		9.5		
0.5 γ adenylic acid.....	0.2	1.1	4.8		4.6		
None.....	0	0				0	2.5
0.05 γ biotin.....	2.5	3.7				6.9	7.6
50 γ adenylic acid.....	0	0				3.7	12.4
None.....	0	0				4.0	12.9
0.05 γ biotin.....	2.0	4.0				6.6	13.2
50 γ adenylic acid.....	0	0				6.6	20.2
None.....		2.9		4.6		6.8	10.5
0.5 γ biotin.....		10.5		16.0		25.6	25.6
50 " adenylic acid.....		3.3		11.0		16.0	33.0
None.....	1.2	2.9		4.6			
0.5 γ biotin.....	4.8	10.5		10.5			
50 " adenylic acid.....	1.2	4.0		11.0			

conditions which enable the cells to gain energy from them to synthesize the coenzyme. That synthesis of the aspartate deaminase coenzyme does occur even in the absence of added substances may be seen from a comparison of the figures for no additions at pH 7 and 4. Such a comparison shows that at pH 7 the cells can synthesize the coenzyme, while at pH 4 this synthesis is either absent or greatly reduced. Biotin stimulation of aspartate deaminase seems not to depend on the pH, although it is usually more pronounced at pH 4 than at pH 7 because of the inability of the cells to synthesize the coenzyme without this vitamin at the former pH. Stim-

TABLE VI

Effect of Various Substances on Deamination of Aspartic Acid by Bacterium cadaveris

Additions per ml	Ammonia nitrogen produced					
	pH 7			pH 4		
	30 min.	60 min.		30 min.	60 min.	
	γ	γ		γ	γ	
None.	4.6	7.5		1.9	3.0	
0.05 γ biotin	9.1	10.9		6.8	7.7	
50 γ adenylic acid	8.9	16.4		2.6	7.5	
50 " adenine	5.0	10.3		2.4	4.0	
50 " guanine	4.6	9.4		1.3	3.4	
50 " ribose	5.7	10.1		2.1	3.0	
50 " xylose	5.2	9.7		2.6	3.6	
	20 min.	40 min.	80 min.	20 min.	40 min.	80 min.
None	13.5	15.3	22.2	2.6	2.6	3.4
0.05 γ biotin	15.5	17.4	21.8	4.0	6.5	11.4
50 γ adenylic acid	15.5	21.0	23.9	2.8	2.3	9.5
0.05 γ biotin + 50 γ adenylic acid	15.5	22.4	28.8	4.0	6.1	16.0
None	8.3	11.6	18.9	1.4	1.8	2.2
0.05 γ biotin	12.1	16.2	25.3	6.9	10.3	14.6
50 γ adenylic acid	10.3	16.4	29.8	1.9	2.3	9.6
0.05 γ biotin + 50 γ adenylic acid	12.6	20.1	33.6	7.3	10.0	17.3

FIG. 3. Effect of pH on biotin and adenylic acid stimulation of aspartic acid deamination in *Bacterium cadaveris*.

ulation by adenylic acid, while differing at pH 7 and 4, is definitely present in both instances as opposed to adenine, guanine, ribose, and xylose. It is noted that, whereas adenylic acid stimulation at pH 7 usually exceeds biotin (Tables V and VI) after a suitable incubation period, at pH 4 the lag period before adenylic acid stimulation is noted is greatly extended and after 80 minutes incubation it has not reached the biotin activity. It is of definite interest to note that in some instances (Table VI and Fig. 3) cells may be stimulated to a greater extent by a combination of biotin and adenylic acid than by either agent alone.

The data of Table VII show that on two occasions cells were obtained which at pH 4 failed to be stimulated by either biotin or adenylic acid

TABLE VII
Effect of Biotin Plus Adenylic Acid on Aspartic Acid Deaminase in Bacterium cadaveris

• Additions per ml.	Ammonia nitrogen produced			
	pH 4		pH 7	
	30 min.	60 min.	30 min.	60 min.
None.	7	7	7	7
0.05 γ biotin	2.8	2.8	7.5	16.0
50 γ adenylic acid	2.8	2.8	12.2	20.2
0.05 γ biotin + 50 γ adenylic acid	2.8	2.8	8.3	24.0
0.05 γ biotin + 50 γ adenylic acid	2.8	8.6	12.6	22.6
None	1.9	1.9	6.3	12.9
0.05 γ biotin	1.9	1.9	11.7	21.1
50 γ adenylic acid	1.9	1.9	6.9	22.2
0.05 γ biotin + 50 γ adenylic acid	1.9	6.5	12.1	23.4

alone, but were markedly stimulated by a combination of biotin and adenylic acid. These same cells at pH 7 were able to synthesize coenzyme, biotin activity and adenylic acid stimulation were typical, and very slight if any additive effect is noted when both biotin and adenylic acid are combined. These data suggest that the third or fourth hypothesis may be valid; namely, that adenylic acid is intimately associated with these deaminases, either having the function of phosphorylating biotin to an active coenzyme form or by chemically combining with biotin to form the coenzyme.

It might be well to consider in some detail the aging process as employed in our studies to obtain a biotin deficiency. Microbiological assays for biotin content in fresh and aged suspensions of *Bacterium cadaveris* have shown a definite reduction in biotin content after aging at pH 4 in phosphate

buffer. Thus in one typical experiment a freshly harvested bacterial suspension contained 10^{-3} γ of biotin per mg. of cell nitrogen, while after aging at pH 4 in M phosphate for 30 minutes at 30° it contained 10^{-5} γ of biotin per mg. of cell nitrogen, or about a 100-fold reduction.

It must be emphasized, as is already evident from some of the data presented, that the degree of biotin deficiency obtained by the aging process varies greatly. At times, although rarely, cells are obtained which are completely unable to deaminate aspartic acid in the absence of added biotin, while at other times, again rarely, cells may be obtained which are unable to deaminate aspartic acid, even upon the addition of biotin. In general the aging technique when carefully controlled will result in cells that show some deficiency in a reaction involving biotin, and biotin stimulation is recorded.

Although the mechanism of the aging process is not known, we feel that it can occur both enzymatically and non-enzymatically, but in living cells the former is probably the case. The mechanism may be a destruction of an active coenzyme form of biotin at pH 4. The following data are in support of this hypothesis. Free biotin can be added at pH 4 and stimulation of the deficient cells occurs, suggesting that biotin can itself be converted to the coenzyme form at this pH. Biotin solutions may be kept at pH 4 without loss in activity, while yeast extract, which can replace biotin in the stimulation of these enzymes, loses its activating effect after 4 to 12 hours at pH 4 at ice box temperatures. In certain instances yeast extract kept at pH 4 will not only lose its stimulatory effect but may show some inhibition. These findings suggest that yeast extract may contain some of the coenzyme form of biotin which is non-enzymatically degraded to an inactive form and in some cases to an inhibitory analogue. While these data do not prove the existence of another active form of biotin, they are suggestive that a coenzyme form does exist. It may be noted that so far all the vitamins which have been shown to function as coenzymes exist in an active phosphorylated form. As to whether or not the aging or splitting phenomenon is an enzymatic one, two other findings should be mentioned. In our experience cells grown in the absence of yeast extract cannot be made biotin-deficient by our technique, and, secondly, living cells can be aged at pH 4, while vacuum-dried preparations have so far given negative results. Both of these findings are suggestive of an enzymatic aging process, since one may postulate that some factor in yeast extract is required for the activity of the enzyme which cleaves the holoenzyme into the apoenzyme and coenzyme, and that this cleaving enzyme is labile to vacuum drying. Certainly if the aging process was merely a matter of

diffusion of a cofactor from the cell into the suspending menstruum, we would expect the process to occur as readily or more so in a vacuum-dried preparation of the same cells.

We have investigated to some degree the optimum conditions for aging and to date can recommend the following as giving the best results in our hands: *growth conditions*, tryptone gives better results than peptone; the addition of formate is advantageous; yeast extract is essential; *aging conditions*, a cell concentration of 1 mg. of N per ml. or less gives best results; phosphate at pH 4 is better than at pH 3 or 5; 20° is better than 10° or 30°.

The data presented in this paper show that both biotin and adenylic acid are concerned with the activation of aspartate, serine, and threonine deaminases. Although the mechanism by which these substances stimulate is not yet entirely clear, the results suggest that biotin exists in a coenzyme form and that adenylic acid is functioning either as a phosphorylating agent of biotin to an active coenzyme or that it combines with biotin to form a coenzyme, possibly similar in structure to diphosphopyridine or triphosphopyridine nucleotide. The studies reported here do not enable one to distinguish between these two or even more possibilities.

It is relevant to add another point regarding biotin and adenylic acid stimulation; namely, whether or not the coenzyme can be assayed. On several occasions microbiological assays for biotin were made on experimental tubes and it was found that biotin could be detected in all instances equal to the amount added plus the amount originally present in the cells. In the case of adenylic acid stimulation no increase in biotin content was found. This may be interpreted as a suggestion that adenylic acid stimulation is an indirect one, having nothing to do with the production of biotin. However, when we consider that in the case of biotin activity no change in biotin content was found, it may be either that the amount of coenzyme formed is so small as not to be detected by our assay methods or that the active biotin coenzyme is not assayed by the method employed.

SUMMARY

Biotin deficiency of several bacterial species was obtained by holding the cells at pH 4 in phosphate buffer at 20–30° for 30 to 60 minutes. Such cells show a markedly decreased ability to deaminate aspartate, serine, and threonine and this activity is restored by the addition of biotin or adenylic acid to washed cell suspensions. Cells similarly treated show no difference in alanine, phenylalanine, methionine, and glutamic acid deaminase activity. Experiments are presented in an attempt to clarify the rôle of biotin and adenylic acid in aspartate, serine, and threonine deaminases.

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ORIGIN AND DISTRIBUTION OF THE HYPERGLYCEMIC-GLYCOGENOLYTIC FACTOR OF THE PANCREAS*

By EARL W. SUTHERLAND AND CHRISTIAN DE DUVE†

(From the Department of Biological Chemistry, Washington University School of Medicine, St. Louis)

(Received for publication, May 20, 1948)

There are many references in the early literature (for a summary see Jensen (1)) to a transient hyperglycemia which is produced in man and animals on intravenous injection of various commercial insulin preparations. This hyperglycemia sets in a few minutes after injection, reaches a maximum in about 5 to 10 minutes, and is then overshadowed by the rapid fall of blood sugar to hypoglycemic levels. Bürger and Brandt (2), in particular, carried out an extensive investigation in order to determine the nature of this hyperglycemic factor. They were unable to separate it from insulin by isoelectric precipitation, alcohol fractionation, or adsorption and concluded that its chemical properties must be very similar to those of insulin.

A separation was, however, effected by crystallizing insulin by the method of Abel *et al.* (3). As shown by several authors (1), this material did not cause an initial hyperglycemia, even on injection into the portal vein. It was then assumed that "impurities" were responsible for the hyperglycemic action of other insulin preparations and little attention was paid to this phenomenon. With the advent of new methods of crystallization, such as that of Scott and Fisher (4), it was apparently taken for granted that they would also lead to a separation of insulin from the hyperglycemic factor. That this is not the case was recently shown by de Duve, Hers, and Bouckaert (5) and confirmed by Olsen and Klein (6). These authors found that, with the exception of one insulin preparation,¹ all others, including crystalline zinc insulin, caused an initial hyperglycemia on intravenous injection in animals.

Shipley and Hümel (7) had demonstrated a glycogenolytic effect of insulin on liver slices *in vitro*, but it was shown by Sutherland and Cori (8) that this was not caused by insulin itself. The Danish insulin,¹ which failed to cause hyperglycemia in animals, was also without effect on liver

* This work was supported by a Rockefeller Foundation postwar assistantship to E. W. S. and by a research grant from Eli Lilly and Company.

† Fellow of the Rockefeller Foundation. Present address, Department of Physiological Chemistry, University of Louvain, Belgium.

¹ Manufactured by the Novo Laboratories in Copenhagen.

slices. The glycogenolytic factor in other insulin preparations was shown to be non-dialyzable, resistant to alkali and cysteine (under conditions which inactivated insulin), and readily destroyed by proteolytic enzymes. Its ability to stimulate glycogenolysis appeared to depend on an intact cell structure. A separation of the glycogenolytic factor from insulin could be effected by a special method of crystallization of the latter.

A study of the distribution and origin of this factor is reported in this paper. Of all tissues investigated only the pancreas and parts of the gastric and intestinal mucosae contained the factor. The distribution followed that of the islet tissue in that there was more factor present in the tail than in the head of dog pancreas. Large amounts of the factor were found in fetal calf pancreas and in sclerosed pancreatic tissue following duct ligation. Finally, normal amounts of the glycogenolytic factor were present in the pancreas of rabbits made diabetic with alloxan.²

A quantitative method of assay of tissue extracts was based on the property of the factor to increase the glucose output of liver slices. Supplementary observations were also made on the changes in blood sugar on injection of tissue extracts into intact animals. The former method has the advantage over the latter that the results are not obscured by the presence of insulin.

Methods

Liver slices were prepared from well fed rabbits by the method previously reported (8). One slice was added to 1.2 cc. of a chloride-phosphate buffer (made by mixing 80 cc. of 0.9 per cent sodium chloride with 20 cc. of 0.1 M potassium phosphate solution, pH 7.4). The slices weighed between 50 and 70 mg. and had a surface area of about 1 sq. cm. Incubation was carried out aerobically in test-tubes, 2.5 × 15 cm., shaken in a Warburg bath at the rate of 110 oscillations per minute. The usual time of incubation was 45 minutes at 37°. The incubation period was terminated by addition of barium hydroxide followed by zinc sulfate and the filtrate was analyzed for glucose. At times glycogen disappearance was measured. Analytical methods have been previously reported (8).

Since the glycogenolytic factor appears to follow insulin through every step of its purification,³ including, in some cases, repeated crystallization, it seemed reasonable to assume that methods which have been specially designed for the quantitative extraction and isolation of insulin from pancreatic tissue would also be suitable for the extraction of the glyco-

² Preliminary observations on the origin of the glycogenolytic factor have been reported (9).

³ It is for this reason that most commercial insulin preparations contain the factor and that ordinary fractionation procedures do not lead to a separation.

genolytic factor. A method described by Best, Haist, and Ridout (10) was used with minor modifications.

The tissues to be analyzed were taken from the living animal under pentobarbital anesthesia, or directly after death, and were frozen immediately. The frozen sample was weighed and then finely minced with scissors in 5 volumes of 75 per cent ethanol containing 0.18 N hydrochloric acid. After extraction overnight in the cold, the residue was separated by centrifugation or passage through gauze, and reextracted for 1 hour with 2.5 volumes of acid alcohol. The two extracts were combined, adjusted to pH 7.5 with ammonium hydroxide, centrifuged or filtered, and the precipitate discarded. The factor (and insulin) was then precipitated by the addition of 1.7 volumes of absolute ethanol and 2.8 volumes of ethyl ether. After standing overnight in the cold, the precipitate was separated by centrifugation and dried *in vacuo*. The dried powder was extracted with isotonic chloride-phosphate buffer (1 cc. per gm. of original tissue), insoluble material was removed by high speed centrifugation, and the clear extract dialyzed against chloride-phosphate buffer before testing.

Adequacy of the method was established by good recovery of known amounts of factor which had been added to inactive tissue. That the factor was not formed from insulin itself in the course of the isolation procedure was shown by addition of insulin preparations free of the factor to inactive tissue.

Routine tests usually comprised two sets of ten slices, each prepared from the same piece of liver. Six of these were used for the titration of unknown samples. Of the remaining four, two served for the determination of the basal glucose output, while two were incubated with an excess of glycogenolytic factor (0.05 mg. of a stock insulin powder) to determine the maximal glucose output. It had previously been established that 0.02 mg. of this reference insulin preparation gave maximal stimulation in this test system. Although the glucose output of the slices varied from animal to animal, added factor never failed to produce stimulation. Frequently the glucose output of the control slices was approximately 7 mg. per gm. per hour, while the output in the presence of an active insulin preparation was 15 mg. per gm. per hour. It has also been shown that there is a graded and reproducible response to different concentrations of the factor and this makes it possible to use the liver test system for quantitative assay of tissue extracts.

Most of the data are presented in terms of per cent of maximal stimulation given by an extract from a stated amount of tissue according to the formula

$$\% \text{ maximal effect} = \frac{x - c}{c_t - c} \times 100$$

GLYCOGENOLYTIC FACTOR

where x is the glucose output of the slice incubated with the unknown, c the glucose output of the control slices (mean of two values), and c_r the glucose output of the slices incubated with the reference insulin powder (mean of two values).

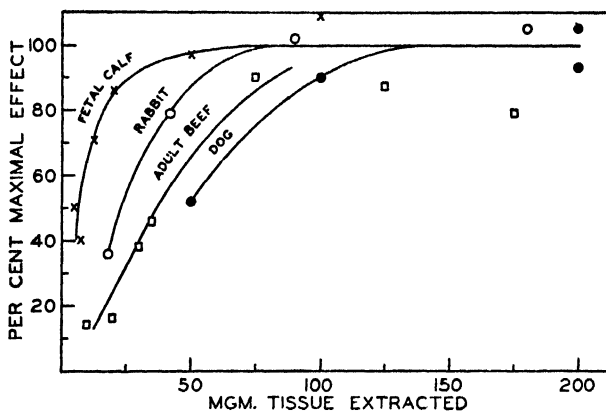


FIG. 1. Glycogenolytic effect of extracts of normal adult pancreas and fetal pancreas on liver slices in per cent of maximal effect obtained by addition of "insulin."

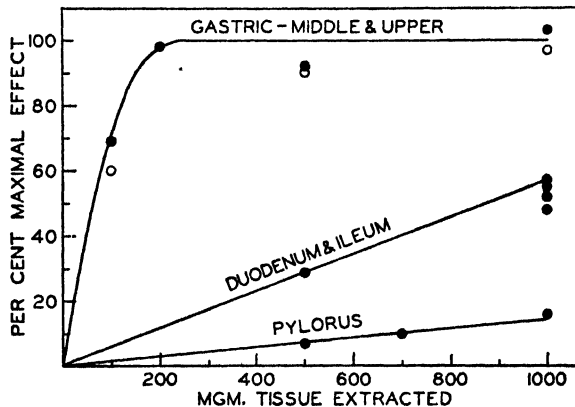


FIG. 2. Glycogenolytic effect of extracts of gastric and intestinal mucosa of dogs on liver slices in per cent of maximal effect obtained by addition of "insulin."

Results

Distribution in Tissues—Assays of pancreatic extracts from the different species are shown in Fig. 1. The extract of rabbit pancreas was obtained from eight pooled specimens, the beef extract from six pooled specimens. Two dog pancreases were extracted separately; the results given represent an average of the results from the two extracts. Fig. 2 shows that con-

siderable amounts of the factor are also present in the upper three-fourths of the gastric mucosa of the dog. The mucosa was separated from the muscular layers before extraction. An extract of about 200 mg. of mucosa gave maximal activity in the test system; it was therefore roughly one-half as active as pancreatic tissue. Small but detectable amounts were present in the duodenum and ileum. Almost none of the factor was found in the pyloric portion of the gastric mucosa. An extract of rabbit stomach also stimulated glucose output from liver slices. However, extracts of the gastric mucosa of other species (hog, beef, sheep) did not cause increased glycogenolysis in liver slices.

Attempts to demonstrate the factor in external secretions of the pancreas, stomach, or in duodenal juice were unsuccessful. A cannula was introduced into the major duct of dogs under pentobarbital anesthesia and the pancreatic secretion collected in tubes chilled on ice. As a control procedure "insulin" was added to one of the tubes. An extract corresponding to about 0.4 cc. of pancreatic secretion was tested on liver slices with negative results. The glycogenolytic factor in the added insulin was recovered satisfactorily. This was probably due to the fact that the pancreatic secretion (as shown by actual determinations) contained large amounts of trypsin inhibitor and little, if any, active form of proteolytic enzymes. Gastric and duodenal juice was collected 40 minutes after feeding 50 gm. of lean meat cooked with 50 cc. of 0.1 M Na_2HPO_4 . The gastric juice was pH 5 and the duodenal juice pH 7. Portions of each were incubated with "insulin" for 40 minutes at 37°. Samples were extracted and tested in amounts representing 0.1 to 1.8 cc. of original sample. A glycogenolytic effect could not be demonstrated in any of the samples. Since the added factor was destroyed by both gastric and duodenal juice, the negative result is not decisive.

In several experiments blood was collected from the pancreaticoduodenal vein, citrated, and chilled. After centrifugation of the blood the plasma was drawn off, lyophilized, and subjected to the usual extraction procedure. In other experiments a pancreas, after ligation of other blood vessels, was perfused through the pancreaticoduodenal artery with warm Ringer's solution saturated with oxygen. The same fluid was allowed to run several times through the pancreas. The perfusates were concentrated ten times and the plasma twenty-five times by the extraction procedure. While apparently positive tests were obtained in some cases, others were completely negative. So far the glycogenolytic factor has not been identified with certainty in these materials. It was noted incidentally that the factor added to blood was destroyed at an appreciable rate.

The glycogenolytic factor could not be demonstrated in other tissues. Those examined with negative results included salivary glands, esophagus,

colon, trachea mucosa, gallbladder, liver, kidney, spleen, skeletal muscle, heart, lung, brain, blood cells, pituitary, thyroid, adrenal medulla, and adrenal cortex. Non-dialyzed extracts of skeletal muscle of several species contained a substance (probably hexose phosphate) from which reducing material was formed during incubation with liver slices. This effect disappeared when the extracts were dialyzed before testing. In the case of adrenal extracts it was necessary to remove epinephrine by dialysis or by

TABLE I
Properties of Glycogenolytic Factor

The tissue extracts were tested for their glycogenolytic effect on liver slices. The results are expressed in per cent of maximal stimulation obtained by addition of "insulin" to control liver slices.

Tissue (dog)	Amount extracted	Additions or treatment	Stimulation
	mg.		per cent
Pancreas	200	None	93
"	100	"	100
"	200	0.05 mg. insulin	87
"	100	0.05 " "	118
"	400	Dialysis	84
"	200	"	105
"	100	"	100
"	300	Incubated, 0.005 mg. chymotrypsin	11
"	400	" 0.1 N KOH 36°, 3 hrs.	70
"	400	Same + 0.05 mg. insulin	104
Gastric mucosa	700	None	104
" "	700	0.05 mg. insulin	122
" "	700	Dialysis	104
" "	200	"	98
" "	500	Incubated, 0.005 mg. chymotrypsin	19
" "	500	" 0.05 " "	13
" "	200	" 0.1 N KOH 36°, 3 hrs.	76

treatment with alkali, because small amounts of epinephrine were found to stimulate glycogenolysis in liver slices.

Comparison of Glycogenolytic Factor in Tissue Extracts and in "Insulin"—The factor extracted from pancreas or gastric mucosa erases the effect of added "insulin;" i.e., when maximal activity was obtained by addition of a tissue extract, addition of the reference insulin powder did not cause a further increase in glycogenolysis. The tissue factor like the factor in "insulin" was non-dialyzable, was readily destroyed by proteolytic enzymes, was resistant to incubation with alkali, and caused hyperglycemia on injection into animals. Table I summarizes some examples of such

experiments. The per cent maximal effect was the same when glycogen disappearance instead of glucose output was measured.

Distribution in Normal Pancreas—The splenic portion or tail of the dog pancreas is readily separable from the middle portion, which is adherent to the duodenum. The middle portion in turn is separable from the head of the pancreas, which usually lies free in the mesentery. When these three parts were extracted separately and assayed, marked differences in content of the factor were found (Fig. 3). The tail contained at least 10 times as much activity as the head of the pancreas, while the middle portion contained intermediate amounts. Histological preparations were made from the same parts of the pancreas that were used for extraction; these showed that there was much more islet tissue in the tail than in the head. This suggested that the factor may originate in islet tissue.

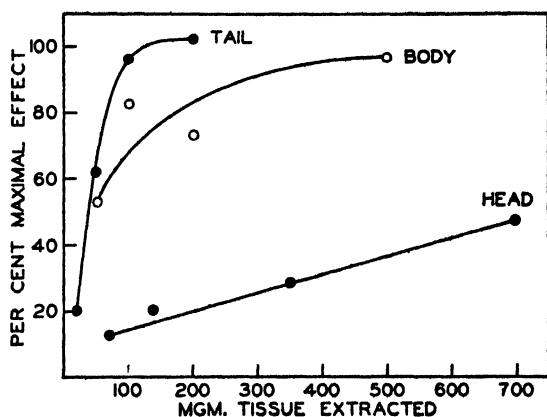


FIG. 3. Glycogenolytic effect of extracts of different parts of the normal dog pancreas on liver slices in per cent of maximal effect obtained by addition of "insulin."

Fetal Calf Pancreas—The fetal calf pancreas contains relatively small amounts of digestive enzymes, even during the later fetal stages. Histologically the ratio of islet tissue to acinar tissue is much higher in the fetus than in the adult, and per unit of weight there is much more insulin in the fetal pancreas than in the adult pancreas. The pancreases of six fetuses approximately 3 months of age were pooled and extracted. As shown in Fig. 1 these contained about 6 times as much factor per unit weight as the extracts of six pooled adult beef pancreases. Pancreases of fetuses approximately 5 and 7 months of age gave similar results.

Duct Ligation—The increased amount of factor in the fetal pancreas reinforced the idea that it was present mainly in the islet tissue. For further evidence, the pancreatic ducts of several dogs were ligated in order

to cause degeneration of the acinar tissue. The sclerosed glands were removed and extracted 6 to 7 weeks after duct ligation. Grossly the glands were small and hard; microscopically the acini had degenerated.⁴ Such glands contained increased amounts of the glycogenolytic factor per unit of weight. Fig. 4 shows the activity of sclerosed pancreas compared to the activity of normal dog pancreas.

Alloxan-Diabetic Rabbits—In order to test the possibility that the glycogenolytic factor was formed in the β cells of the islet tissue, use was made of the destructive effect of alloxan on these cells. Rabbits were injected intravenously with alloxan; animals considered diabetic had hyperglycemia, glycosuria, lipemia, and in the cases measured had very low liver glycogen.

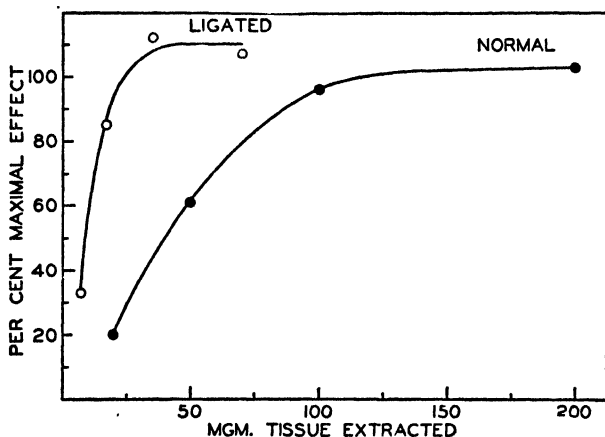


FIG. 4. Glycogenolytic effect of extracts of the tail of normal and ligated pancreas on liver slices in per cent of maximal effect obtained by addition of "insulin."

Pancreatic extracts prepared from these animals contained the glycogenolytic factor in normal amounts, as may be seen from Fig. 5. That the treatment with alloxan had resulted in the destruction of β cells is indicated by the absence of insulin in the pancreatic extracts (see below).

Animal Injection Experiments—A number of the extracts were injected

⁴ It has been found that the method used for the extraction and precipitation of the glycogenolytic factor yields an active preparation of trypsin inhibitor. Trypsin inhibitor was measured by its ability to inhibit the digestion of azocasein by trypsin (11). Following duct ligation the trypsin inhibitor concentration per unit weight fell sharply (while the concentration of the glycogenolytic factor rose). The assay of trypsin inhibitor therefore confirms the histological data regarding acinar degeneration. Other observations were that fetal calf pancreas contained less inhibitor than the adult pancreas and that the external secretion of the pancreas contained a high concentration of inhibitor. Most of the assays of trypsin inhibitor were carried out by Mr. Robert Haynes.

intravenously into rabbits and the changes in blood sugar were followed. This served not only to characterize the material by demonstrating a

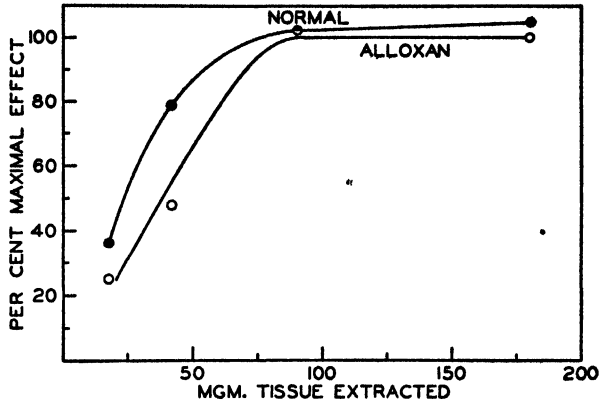


FIG. 5. Glycogenolytic effect of pancreatic extracts from normal and alloxan-diabetic rabbits on liver slices in per cent of maximal effect obtained by addition of "insulin."

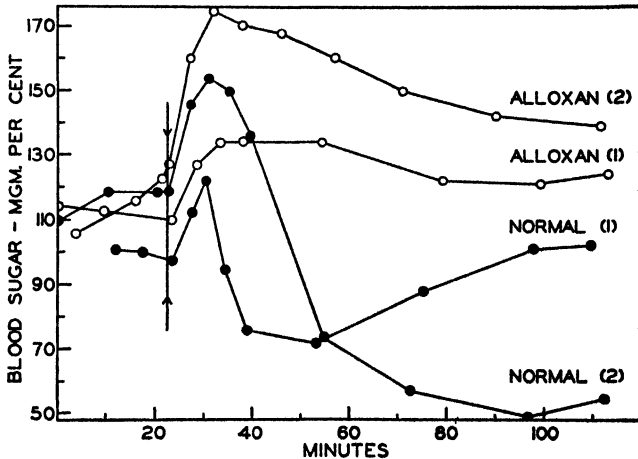


FIG. 6. Blood sugar changes following intravenous injection of pancreatic extracts of normal and alloxan diabetic rabbits into normal rabbits. Curves 1 resulted from the injection of an extract representing approximately 0.5 gm. of pancreas; Curves 2 from 1.5 gm. of pancreas.

hyperglycemic response, but also gave a rough estimate of the insulin content of the extracts. Fig. 6 shows the results obtained from injection of extracts prepared from normal rabbit pancreases or from the pancreases of alloxan-diabetic rabbits. The extracts from normal animals produced

an initial hyperglycemia which was soon followed by hypoglycemia. These curves are typical of those seen when certain commercial insulin preparations are injected intravenously. The extracts from the pancreases of alloxan-diabetic animals produced a prolonged hyperglycemia which was

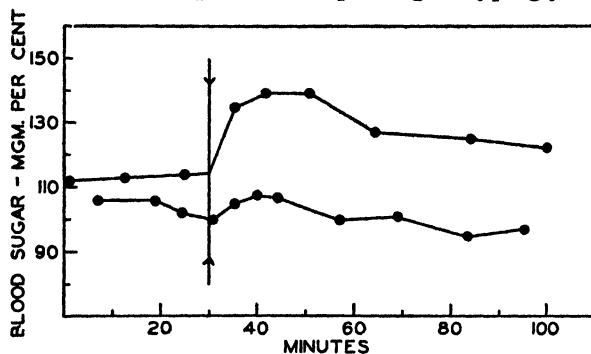


FIG. 7. Blood sugar changes following intravenous injection of extracts of dog gastric mucosa into normal rabbits. The upper curve corresponds to the injection of an extract from 5 gm. of mucosa, the lower curve from 1.0 gm.

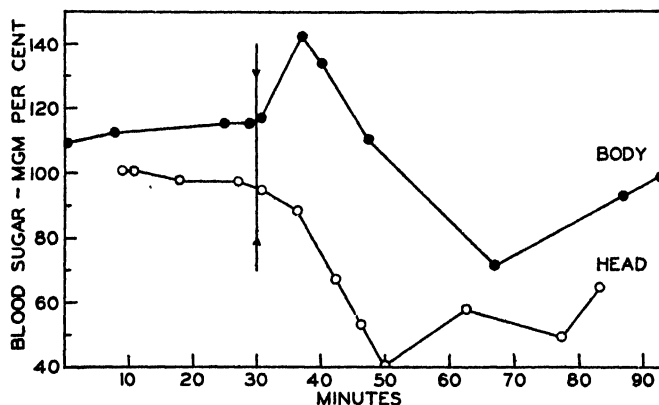


FIG. 8. Blood sugar changes following intravenous injection of extracts of the body and head of normal dog pancreas into rabbits. In both cases, the extract represented 1.0 gm. of pancreas.

not followed by hypoglycemia. The injection of these extracts illustrates the action of the hyperglycemic factor when it is unopposed by the hypoglycemic action of insulin. Extracts of gastric mucosa also caused prolonged hyperglycemia without subsequent hypoglycemia (see Fig. 7).

Fig. 8 shows the blood sugar curves after injection of extracts from the middle portion (body) and the head of normal dog pancreas. Extracts from the middle portion produced the characteristic initial hyperglycemia

followed by hypoglycemia, while extracts from the head produced hypoglycemia without initial hyperglycemia. This confirms the results obtained in the assay of these extracts on liver slices relative to the distribution of the hyperglycemic factor in different parts of the pancreas (see Fig. 3).

DISCUSSION

Experiments designed to localize the site of formation of the hyperglycemic-glycogenolytic factor in the pancreas may be summarized as follows. The distribution in different parts of the normal dog pancreas, the high concentration in the fetal calf pancreas, and the presence of increased amounts in the sclerosed pancreas following duct ligation constitute strong evidence for production of the factor by islet cells. This is narrowed down further by the observation that the β cells of the islet tissue can be destroyed by alloxan without causing a diminution of the factor in the pancreas.

One might therefore conclude that the α cells are the site of formation of the glycogenolytic factor. However, the presence of an apparently identical factor in the upper two-thirds of the gastric mucosa of the dog raises the question of specificity, unless it were shown that the stomach mucosa contains cell types related to the α cells of the pancreas.⁶ Similarly, one might be tempted to conclude that the glycogenolytic factor is a new hormone of the pancreas, but this seems unwarranted in the absence of a clear cut demonstration that it is secreted into the blood stream and participates in the regulation of the blood sugar level.

SUMMARY

1. A glycogenolytic factor apparently identical with that present in commercial insulin preparations was shown to be present in extracts of pancreatic tissue from several species. It was also found in considerable amounts in the upper three-fourths of the gastric mucosa of the dog but not in pyloric mucosa. Small amounts were present in the duodenum and ileum. The gastric mucosa of other species (pig, sheep, cattle) contained none or only traces of the glycogenolytic factor. The method of extraction and purification was similar to that used for insulin. Quantitative assays were based on the property of the factor to increase glycogenolysis in liver slices.

⁶ Tehver (12) reported that certain argentophil cells, which occur in small numbers throughout the gastrointestinal tract, are particularly numerous in the fundus mucosa of the dog, while the pyloric mucosa is relatively free of them. Other species including pig, sheep, and cattle show few of these cells in the gastric mucosa. Moreover, several investigators (13, 14) have concluded that certain pancreatic cells which stain with silver are identical with the α -cells. The distribution of the glycogenolytic factor is apparently closely related to the distribution of these argentophil cells.

2. It has not been possible to demonstrate this factor in any other tissue or in the external secretions of the digestive tract.

3. The glycogenolytic factor was unequally distributed in different parts of the dog pancreas and followed roughly the distribution of islet tissue. The tail (or splenic portion) contained the highest concentration, while the head contained very little. This was shown by assay of extracts on liver slices and by injection into intact animals.

4. The factor was present in large amounts in the fetal calf pancreas. The sclerosed pancreatic tissue following duct ligation contained an increased amount per unit weight.

5. Roughly normal amounts of the glycogenolytic factor were found in the pancreas of alloxan-diabetic rabbits. Extracts from such pancreases produced prolonged hyperglycemia without subsequent hypoglycemia when injected intravenously into normal rabbits.

6. The experiments suggest that the glycogenolytic factor of the pancreas originates in the α cells of the islet tissue.

The authors are deeply indebted to Dr. C. F. Cori for his helpful advice and criticism throughout this work. The technical assistance of Miss Dolores Barta is gratefully acknowledged.

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THE COMBINATION OF INSULIN WITH THIOCYANATE IONS

By ELLIOT VOLKIN*.

(From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina)

(Received for publication, May 7, 1948)

Measurements of the electrophoretic mobilities of insulin reported by Hall (1, 2) are limited to regions higher than pH 7 and lower than pH 4.5, since the protein is practically insoluble in common buffer solutions of the intermediate pH range. While it has been possible to determine the electrophoretic mobility of adsorbed insulin and of suspensions of insulin crystals in the isoelectric range by the microscopic method (3), strict comparison of mobility data obtained under these two different experimental conditions is not always permissible (4). Both sets of data, however, indicate that the isoelectric point of insulin lies within the range of pH 4.9 to 5.9.

Attempts have been made in the present study to increase the solubility of insulin in its isoelectric region sufficiently to afford determinations of the electrophoretic mobility in this range by the moving boundary method. Of various ions of the lyotropic series that have been tested, thiocyanate was found to exert a marked peptizing effect. The lower pH limit of solubility just sufficient for mobility measurements was approximately pH 5, the solubility increasing with increasing pH. In the presence of thiocyanate, insulin remained insoluble, however, at all pH regions below 5, including those in which it is soluble in the absence of this ion, *i.e.* pH 2.

The electrophoretic data reported in this paper, together with the observed effect of thiocyanate on the solubility of insulin, suggest an interaction of this anion with certain basic groups of the protein. Considerable evidence has already been given for the binding by proteins of fatty acid anions (5-8), anionic detergents (9-13), and even anions of common buffer salts (14-16). Although some of these combinations may occur with the non-polar residue of the anion (17-18), all of them will be reflected by measurable changes in electrophoretic mobilities.

EXPERIMENTAL

Electrophoretic measurements were carried out at 1° with the Tiselius electrophoresis apparatus equipped with the Thoevert-Philpot-Svensson optical system (19). Only the mobilities of the descending boundaries were

* Present address, Division of Biology, Clinton National Laboratories, Oak Ridge, Tennessee.

considered in this work. The time intervals between the initial and final photographs were generally 3 hours, and the potential gradients were within the range of 1.9 to 2.0 volts per cm.

The crystalline zinc insulin used in this work was obtained through the courtesy of the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis.

One series of measurements was carried out in buffer solutions containing 0.15 M NaCNS, the other in buffer solutions containing 0.15 M NaCl. Citrate, phosphate, and veronal buffers were employed. The citrate buffers were prepared by dissolving calculated quantities of citric acid and the desired amounts of NaCNS or NaCl, respectively, in 95 per cent of the final volume of solution. This was titrated with NaOH to the desired pH with the aid of a Beckman pH meter, and then diluted with water to the final volume. The phosphate buffers were prepared by dissolving NaH_2PO_4 and Na_2HPO_4 in the desired ratio, together with NaCNS or NaCl respectively, in water and diluting to volume. In a like manner, the veronal buffers were prepared from diethylbarbituric acid and its sodium salt. The pH values of all buffer solutions were checked with the Beckman pH meter after their preparation.

The total ionic strength of all buffer solutions containing either 0.15 M NaCNS or 0.15 M NaCl was 0.18 ± 0.01 .

The crystalline zinc insulin was dissolved in 20 cc. of buffer solution and allowed to dialyze against 500 cc. of buffer for 24 hours at 4°. The final protein concentration never exceeded 0.2 per cent. Because of the low protein concentration and high ionic strengths, the δ and ϵ boundaries resulting from electrophoresis were kept at a minimum.

Results and Interpretation

Homogeneity of Crystalline Zinc Insulin in Buffer Solutions—A photograph of a typical electrophoretic experiment is shown in Fig. 1. A single, practically symmetrical, boundary is evident, indicating a high degree of homogeneity. The δ boundary is negligible. These characteristics have been found in all experiments, regardless of pH and buffer composition.

Electrophoretic Mobility in Presence of Thiocyanate—Fig. 2 represents a plot of the electrophoretic mobility of insulin in buffers containing NaCl or NaCNS *versus* pH. Because of the limited solubility of the protein in buffer containing 0.15 M NaCl, measurements had to be confined to regions above pH 6.8 and below pH 4.2. In buffers containing 0.15 M NaCNS, the protein is soluble at pH 5.2 and higher but remains insoluble at all pH values lower than 5, including pH 2 at which pH it is readily soluble in buffers of the NaCl series.

It is evident from comparison of the two curves that, at equivalent pH

values, the anodic mobilities of the protein in the presence of 0.15 M NaCNS are markedly increased over those observed in the presence of 0.15 M NaCl.

FIG. 1. Descending boundary of crystalline zinc insulin in phosphate buffer containing 0.15 M NaCNS, after 3 hours migration. The total ionic strength was 0.18, the pH was 6.62, and the protein concentration was approximately 0.1 per cent. The sharp peak at the left is the starting boundary.

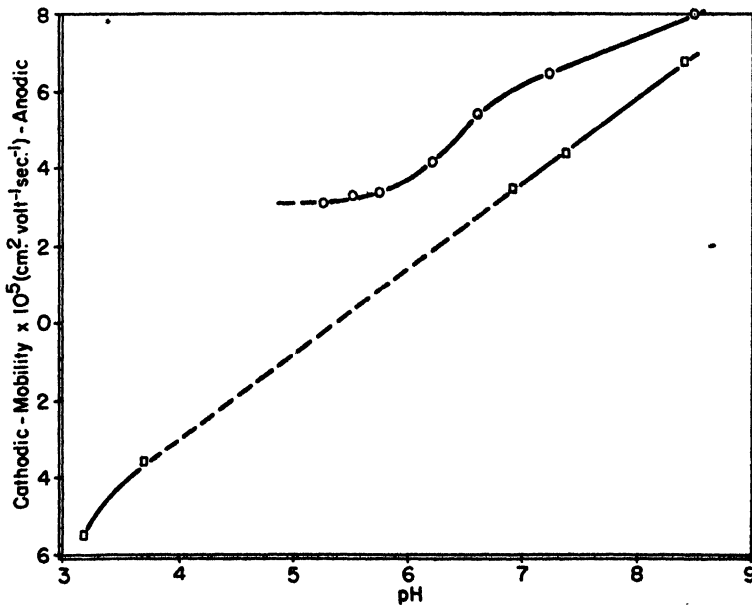


FIG. 2. A plot of the mobilities *versus* pH of insulin in buffers containing 0.15 M NaCNS (○) and in buffers containing 0.15 M NaCl (□). The curves connecting the points were drawn by visual inspection. The region in which the protein is insoluble in the buffer solution containing NaCl is indicated by the broken line.

In the pH region 5.2 to 5.8, the region in which insulin has been estimated to be isoelectric (1-3, 20), the protein, in the presence of 0.15 M NaCNS, still retains an anodic mobility of 3.0 to 3.5 units.

It seems apparent that the interaction between the insulin and thiocyanate causes an increase in the net negative charge of the protein, with the result that under the conditions of these experiments the protein remains negatively charged at all pH values at which it is soluble.

Estimation of Extent of Binding of Thiocyanate—An attempt has been made to correlate the pH-mobility curve of insulin in the presence of thio-

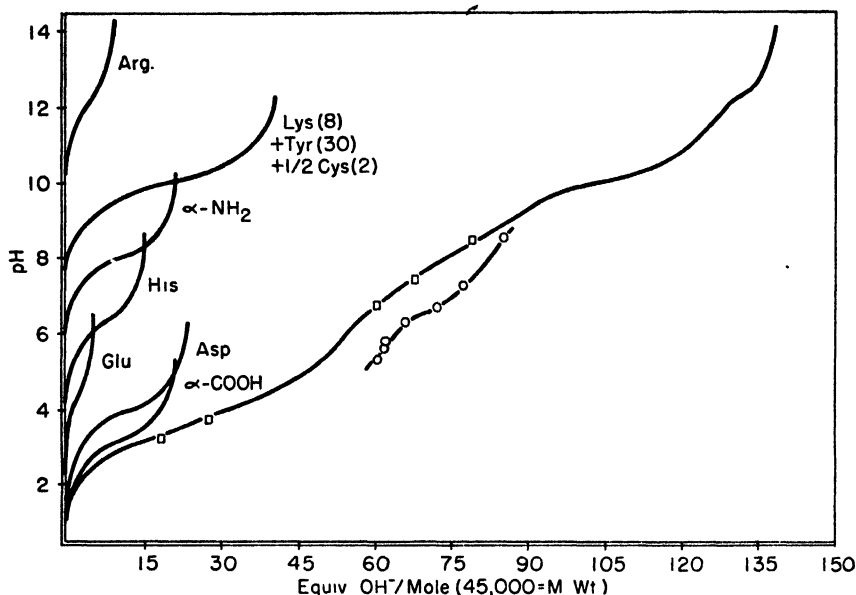


FIG. 3. Comparison of the theoretical titration curve of insulin calculated from analytical data (22) with the mobility data of insulin in buffer solutions containing 0.15 M NaCl and 0.15 M NaCNS. The large curve running diagonally across the graph is the theoretical titration curve, calculated by summation of the titration curves of the individual ionizable amino acids (shown at the left). □ represents the mobilities of the insulin in buffers containing 0.15 M NaCl adjusted to the titration curve with an empirical factor; ○, the mobilities of the insulin in 0.15 M NaCNS, adjusted with the same empirical factor.

cyanate to the titration curve of the protein, and to estimate thereby the extent of binding of thiocyanate ions. Although titration data are available (*cf.* (21)), the insolubility of insulin in the isoelectric region, pH 4 to 7, precludes the use of these data for the present purpose. The analytical data by Brand (22), however, can be employed to calculate a theoretical titration curve for insulin. Using the data for the number of ionizable amino acid residues in the insulin molecule of molecular weight 45,000 and the mean estimated pK values for the ionizable groups as they occur in protein, given by Cohn and Edsall (21), one can construct a theoretical titration curve such as that given in Fig. 3. This curve has been con-

structed with the considerations that (a) at the start of the titration the protein has already bound its capacity of acid, and (b) the titration with base proceeds until the maximum base binding has been reached. At the left of Fig. 3, the titration curves for the individual ionizable amino acids in insulin are shown, including 9 arginine, 8 lysine, 30 tyrosine, 2 cysteine, 15 histidine, 23 aspartic, 5 glutamic, 21 terminal α -amino, and 21 terminal α -carboxyl residues per molecule. It has been assumed that the 21 residues of cystine in the insulin molecule yield no free ionizable groups. The large titration curve in the center of Fig. 3 is a plot of the total base bound, obtained by summation of the base bound by the individual amino acids, at various pH values, over small increments of pH. Comparison of this theoretical titration curve with the experimental titration curve (21) reveals the two to be superimposable from pH 2 to 12, exclusive of the region pH 4 to 7 for which no titration data are available.

The usual empirical procedure for comparing titration curves with pH-mobility curves consists in shifting the titration curve along the pH axis to the point at which 0 equivalent of acid or base bound coincides with isoelectric point (4, 23). Since electrophoretic mobilities near the isoelectric point could not be determined, recourse was had to an empirical conversion factor, which may be defined as the change in base equivalents bound corresponding to 1 unit change in mobility (1×10^{-6} sq. cm. sec.⁻¹ volt⁻¹). This conversion factor was determined by selecting two adjacent, experimentally determined mobility values for reference. The difference in equivalents of base bound at the two pH values corresponding to the reference mobilities was divided by the difference in mobility units, and other points on the titration curve were compared to the mobility curve with this factor by the following relations: Let u_1, u_2, u_3 , etc., be the mobilities at pH₁, pH₂, pH₃, etc.; e_1, e_2, e_3 , etc., are the corresponding equivalents of base bound. The conversion factor, F , is then $F = (e_1 - e_2)/(u_1 - u_2)$. The complete titration curve may then be calculated from this factor and the experimentally determined mobilities. For instance, $e_3 = (u_3 - u_1) \times F + e_1$. In the present instance, the mobilities at pH 6.92 and 7.38 in 0.15 M NaCl and the corresponding equivalents of base bound were used as reference for the calculation of F .¹ The equivalents of base bound, calculated from the mobilities of insulin in the presence of 0.15 M NaCl at pH 8.42, 3.70, and 3.18 (e_3, e_4, e_5), are indicated on the titration curve of Fig. 3, together with the two reference points (e_1 and e_2 at, respectively, pH 6.92 and 7.38) by the

¹ The value of F calculated from the present experimental data is 5.15. Calculation with the aid of the Henry theory (4, 23), the corrections subsequently introduced by Abramson *et al.* (4) for the "average" radius of the ions in the ion atmosphere being omitted, yields a value of $e/u = 2.53$. The magnitude of the discrepancy between these two values is comparable to that previously found by Longworth (23) for analogous calculations for egg albumin.

squares. It can be seen that the calculated points fit exceedingly well on the theoretical titration curve.

The same factor was used for the calculation of equivalents of base bound from mobilities determined in the presence of 0.15 M NaCNS. In this manner the change in base equivalents bound corresponding to the difference in mobility of insulin in the presence of 0.15 M NaCNS and 0.15 M NaCl, respectively, was determined. It can be seen that the points obtained for insulin in the presence of 0.15 M NaCNS are displaced in the direction of greater base binding.

From the titration curves of the individual amino acid residues it can be determined that at pH 5.8 the number of positively and negatively charged groups is equal. This pH, at which the net charge is zero, may be considered as the isoionic point. At the same pH, the equivalents of base bound by insulin in the presence of NaCNS exceed by 9 or 10 equivalents the amount of base bound by insulin in the presence of 0.15 M NaCl.

One may speculate that the binding of thiocyanate ions takes place through an electrostatic linkage with the strongly basic guanidino groups of the 9 arginine residues or with the ϵ -amino groups of the 8 lysine residues in the insulin molecule. This is suggested by the convergence of the mobility curves above pH 8 and by the effect of thiocyanate on the solubility of insulin in the acid pH region. It is of interest to note that the number of anionic detergent molecules bound by serum albumin in the first complex (11, 12) likewise coincides with the number of arginine residues in this protein. More direct evidence than that presented here must be forthcoming to prove this hypothesis.

The author wishes to record his appreciation to Dr. Hans Neurath and Dr. George W. Schwert for their helpful advice throughout this study. This work has been supported by grants from the Rockefeller Foundation, from Eli Lilly and Company, and from the United States Public Health Service, National Institute of Health, Division of Research Grants and Fellowships.

SUMMARY

The electrophoretic mobility of crystalline zinc insulin in buffers containing 0.15 M NaCNS and 0.15 M NaCl, respectively, has been investigated.

In the presence of thiocyanate, the solubility of insulin is sufficiently increased to afford mobility measurements with the moving boundary method within the isoelectric range. The increased electrophoretic mobility over that observed in the presence of NaCl corresponds to an increase in net negative charge, suggesting association of thiocyanate with basic groups of the protein.

Comparison of mobility data with a theoretical titration curve of insulin calculated from summation of the base-binding capacities of the constituent amino acid residues reveals that at the isoionic point of the protein a net negative charge of approximately 9 or 10 is retained in buffers containing 0.15 M NaCNS.

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THE SYNTHESIS OF AMINO ANALOGUES OF INOSITOL (INOSAMINES)*

By HERBERT E. CARTER, R. K. CLARK, JR., BETTY LYTTLE,† AND
G. E. McCASLAND‡

(From the Division of Biochemistry, Noyes Laboratory of Chemistry, University of Illinois, Urbana)

(Received for publication, May 17, 1948)

In the course of work on the structure and configuration of streptamine (*meso*-1,3-diamino-2,4,5,6-tetrahydroxycyclohexane) it seemed of interest to investigate the properties of the corresponding monoamino compounds (2,3,4,5,6-pentahydroxycyclohexylamines). We wish to propose the generic name *inosamine* for these amino analogues of inositol, since they are related to the inososes in the same way that glucamine is related to glucose.

As one approach to the synthesis of inosamines, we have investigated the reduction of the phenylhydrazone (1, 2) and the oxime (2) of *scyllo*-inosose and the phenylhydrazone (3) of *dl*-*epi*-inosose. Each of these substances was hydrogenated readily at high pressures with Raney's nickel as the catalyst, and the main product in each case consisted of an inosamine.

The inosamines can exist theoretically in eight *meso* and twelve *dl* forms. However, the configuration of the five hydroxyl groups in each of the above inosose derivatives is fixed and known. Thus, unless unexpected C—O bond inversions occur during the hydrogenation, *scyllo*-inosose phenylhydrazone (I) or oxime (II) should give rise to not more than two inosamines (III, IV), each of which should have a *meso* configuration; and *dl*-*epi*-inosose phenylhydrazone (V) should yield a maximum of two racemic forms (VI, VII).

Experimentally, the inosamine from *scyllo*-inosose was obtained in two epimeric forms, each of which has been isolated in a pure state. Since the configuration of each epimer remains uncertain at the C—N bond, we have temporarily adopted the purely arbitrary names¹ *inosamine-SA* and

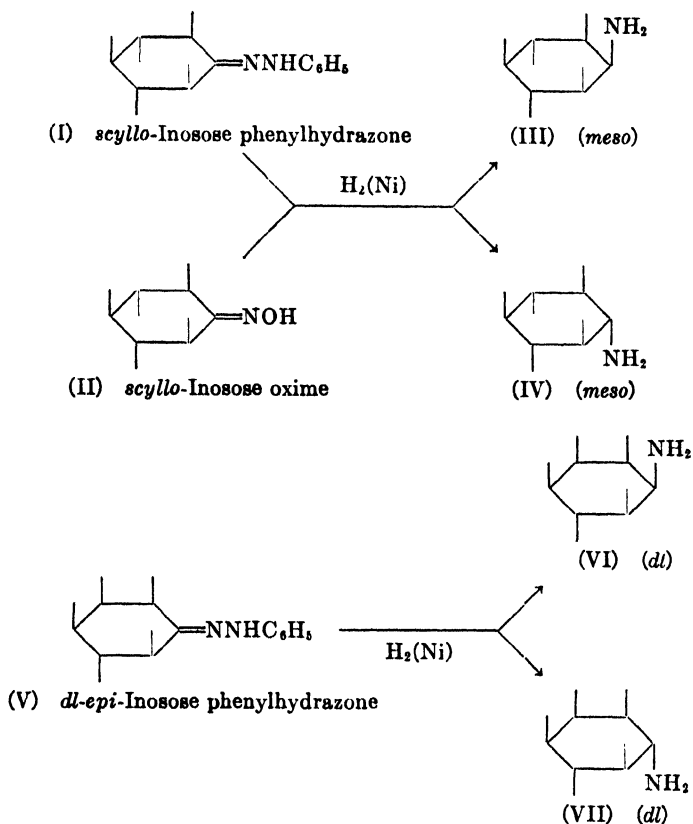
* The authors wish to thank the Abbott Laboratories, Eli Lilly and Company, Parke, Davis and Company, and The Upjohn Company for a generous grant in support of this work. Part of the material in this paper was taken from the thesis submitted by R. K. Clark, Jr., to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry.

† Present address, Davenport, Iowa.

‡ Postdoctorate Research Assistant in Chemistry.

¹ The use of such names as "*scyllo*-inosamine" or "*epi*-inosamine" is undesirable

inosamine-SB for these compounds. Of the two possible *dl*-inosamines from *dl-epi*-inosose, only one has thus far been obtained, and it is designated *dl-inosamine-EA*.



The fact that both the oxime and phenylhydrazone of *scyllo*-inosose give the same two inosamines on reduction indicates that no isomerization occurs in the preparation of the oxime despite the highly alkaline conditions employed (2).

Separation of Epimeric Inosamines

The hydrogenation product of *scyllo*-inosose phenylhydrazone or oxime is a mixture of inosamine-SA and inosamine-SB. Repeated recrystallization of this mixture from water yields pure inosamine-SA. This compound,

because only one of each pair of epimers has the "*scyllo*" or "*epi*" configuration, respectively, and it is not known which one. (See the comments on nomenclature in our previous paper (2).)

like the other inosamines, cannot be characterized by melting point, but its purity can be recognized by the attainment of constant solubility on repeated recrystallization.

In order to obtain inosamine-SB, the mixed epimers were converted to the mixed hexaacetyl derivatives. The latter were very difficult to separate. However, methanolic ammonia readily converts the hexaacetyl inosamines to N-acetyl inosamines. Very fortunately, while inosamine-SB is *more* soluble in water, its N-acetyl derivative is *less* soluble in ammoniacal or aqueous methanol, with respect to inosamine-SA. The reversal of solubilities affords a convenient basis for obtaining either epimer in a pure state.

The stereochemistry of the inosamines is being studied further.

EXPERIMENTAL

Compounds Derived from scyllo-Inosose

Inosamine-SA from Phenylhydrazone—A 36 gm. portion of *scyllo*-inosose phenylhydrazone (1, 2) was hydrogenated in methanol at 100 to 150 atmospheres and 130° with Raney's nickel catalyst. The hydrogen uptake reached the theoretical in a few hours. The mixture was filtered. The methanolic filtrate on vacuum distillation gave aniline but no inosamine. The precipitate was repeatedly extracted with boiling water (total 600 to 700 ml.) until only catalyst remained. Vacuum distillation of the extract yielded 10.1 gm. of solid residue. Recrystallization from boiling water (norit) gave colorless crystals. Recrystallization was repeated until the water solubility reached a constant value of about 0.7 gm. at 25° and 5.0 gm. at 100° per 100 ml. of solvent. 4.1 gm. of pure inosamine-SA were thus obtained. The compound crystallizes as colorless prisms which decompose² gradually above 240°. The crystals give an alkaline solution in water and are very soluble in dilute hydrochloric acid.

The mother liquors were reserved for preparation of N-acetyl inosamine-SB. The early mother liquors which are saturated with respect to inosamine-SB as well as SA (maximum enrichment in inosamine-SB) are particularly suitable for this purpose.

Attempts to reduce *scyllo*-inosose phenylhydrazone to inosamine by the method used by Feofilaktov (4) for phenylhydrazones of α -keto acids proved unsuccessful.

Inosamine-SA from the Oxime—A 23 gm. portion of freshly prepared, dry *scyllo*-inosose oxime (2) was dissolved in 150 ml. of concentrated aqueous ammonia,³ and immediately hydrogenated at 100 to 150 atmos-

² All melting points were determined on the K $\ddot{o}$ fler micro block.

³ Aqueous ammonia was used to repress secondary amine formation. In an earlier hydrogenation, with distilled water as solvent, a small amount of secondary amine

pheres and room temperature with Raney's nickel catalyst. The uptake of hydrogen was complete in about 2 hours.

After filtration, the precipitate was extracted with 800 to 1200 ml. of boiling water. The combined filtrates on vacuum distillation to dryness (foaming near the end; use a large flask) yielded 19.3 gm. of solid residue.

Recrystallization of this residue from water gave 6.3 gm. of nearly colorless crystals. These were recrystallized from a minimum amount (120 ml.) of water (norit), yielding 4.5 gm. of colorless prisms of pure inosamine-SA.

The mother liquor from the first recrystallization was treated with norit and concentrated to 50 to 60 ml. On cooling, 3.7 gm. of a light tan powder (mixture of inosamine-SA and SB) were obtained. The filtrate from this material on evaporation gave a tarry substance, which has not been further investigated.

In an earlier experiment, 6.0 gm. of oxime were hydrogenated in 150 ml. of solvent, and extraction of the catalyst with boiling water was omitted. The crude mixed inosamines obtained weighed 3.9 gm., and gave the following analysis.

$C_8H_{13}O_2N$ (179.17). Calculated. N 7.82
Found. " 7.39 (Kjeldahl), 7.82 (Van Slyke)

Mixture of Hexaacetylinosamine-SA and SB—Direct acetylation of 1 gm. of crude mixed inosamines (from the phenylhydrazone) with acetic anhydride-sodium acetate gave after recrystallization 1.1 gm. of colorless needles, with a melting range of 235–247°, unchanged by repeated recrystallizations.

$C_{18}H_{26}O_{11}N$ (431.39). Calculated. C 50.11, H 5.84, N 3.25
Found. " 50.07, " 5.74, " 3.23 (Dumas)
" 3.54 (Kjeldahl)

Saponification equivalent (O-Acetyl). Calculated, 86.3; found, 86.0, 86.6

Similar acetylation of 2.1 gm. of crude mixed inosamines from the oxime gave 2.7 gm. of colorless needles of melting range 238–252°, unchanged by repeated crystallizations from ethanol or dilute acetic acid.

$C_{18}H_{26}O_{11}N$ (431.39). Calculated. C 50.11, H 5.84, N 3.25
Found. " 50.05, " 5.99, " 3.14

Hexaacetylinosamine-SA—A mixture of 1.4 gm. of pure inosamine-SA, 28 ml. of acetic anhydride, and 0.2 gm. of fused sodium acetate was re-

was formed. Treatment of the latter with excess acetic anhydride gave a compound, colorless needles, m.p. 285°, which is apparently the (not quite pure) undecaacetyl derivative. $C_{24}H_{40}O_{21}N$ (803.71). calculated, C 50.80, H 5.64, N 1.74; found, C 49.53, H 5.68, N 1.2 (Dumas), N 1.78 (Kjeldahl).

fluxed for 1 hour, and then vacuum-distilled to dryness. The residue was washed repeatedly with water and dried at 100° *in vacuo* over phosphorus pentoxide. The colorless needles (3.4 gm.) obtained melted sharply at 259–260°; after resolidification the sample melted at 256–260°.

A 1 gm. sample was recrystallized from 50 ml. of absolute ethanol. After drying the crystals as above, 0.8 gm. of colorless needles, m.p. 260–261°, was obtained. (At 245–247° the substance undergoes a change in crystal form, without appreciable liquefaction.)

Reacetylation of purified N-acetylinoamine-SA, by the above procedure, gave a product with identical properties, further indicating the homogeneity of the hexaacetyl derivative.

N-Acetylinoamine-SA—0.5 gm. of pure hexaacetylinoamine-SA was added to 20 ml. of absolute methanolic ammonia (saturated at 25°). A clear solution was obtained after 2 minutes stirring. After 24 hours, the solution was decanted from the crystals (125 mg.) which had separated, and vacuum-distilled to dryness. The combined solid residues were washed with methanol, leaving 215 mg. of colorless irregular crystals which melted with decomposition at 242–248° (rapid heating).

A sample recrystallized from 80 per cent methanol melted with decomposition at 246–248° (rapid heating).

$C_8H_{14}O_6N$ (221.21). Calculated. C 43.43, H 6.84, N 6.33
Found. " 43.33, " 6.79, " 6.42

Inosamine-SA Hydrochloride—Pure hexaacetylinoamine-SA (3.0 gm.) was refluxed with 40 ml. of 6 N hydrochloric acid for 2 hours. The clear solution was vacuum-distilled to dryness. The residue (1.5 gm.) was recrystallized from 20 ml. of water-ethanol-acetone (1:2:1), giving 1.3 gm. of the monohydrate as colorless needles melting at 235–237° (change in crystal form at 192°). The monohydrate is stable when vacuum-dried at 80°.

$C_8H_{14}NO_6Cl \cdot H_2O$ (233.66). Calculated. C 30.83, H 6.90, N 6.00
Found. " 30.99, " 7.10, " 6.01

N-Acetylinoamine-SB—A 6.0 gm. portion of mixed epimeric inosamines from the phenylhydrazone (maximum enrichment in inosamine-SB) was acetylated by the above procedure. The crude, dry hexaacetyl derivative (9.0 gm.) was dissolved in 200 ml. of absolute methanolic ammonia. After 24 hours the colorless leaflets which had separated were collected and washed with methanol. The crystals (2.2 gm.) were pure N-acetylinoamine-SB, melting at 289–291° with decomposition.

The filtrate yielded 2.4 gm. of a discolored powder, mainly the SA epimer, which decomposed at about 240°.

A similar mixture of epimeric inosamines from the oxime (3.7 gm.) on

acetylation yielded 8.1 gm. of crude hexaacetyl derivative. A 1.7 gm. portion of the hexaacetyl derivative was treated with 70 ml. of absolute methanolic ammonia. The reaction product (0.70 gm.) was recrystallized from aqueous methanol, giving 0.44 gm. of colorless leaflets, melting sharply with decomposition at 286–288°.

Attempts to obtain further pure product (inosamine-SA or SB) from the mother liquors were unsuccessful.

$C_8H_{16}O_4N$ (221.21):	Calculated.	C 43.44, H 6.84, N 6.33
	Found.	" 43.36, " 6.46, " 6.43
		" 43.28, " 6.55

The product is very soluble in water, insoluble in methanol, and slightly soluble in aqueous or ammoniacal methanol.

Hexaacetylinosamine-SB—A 200 mg. portion of pure N-acetylinoamine-SB was refluxed with 4.0 ml. of acetic anhydride and 10 mg. of fused sodium acetate for 1 hour. On cooling, colorless blades (223 mg.) crystallized from the solution. These were collected, washed with water, and dried at 100° *in vacuo*.

On heating, the clear, colorless blades became opaque at about 200°, and smaller needles gradually replaced them, apparently by sublimation. At 284° the needles changed to more massive crystals, and at 299–301° these melted completely. This complex melting behavior, perhaps representing changes to dimorphic forms, was quite reproducible and unchanged by further purification.

$C_{18}H_{26}O_{11}N$ (431.39).	Calculated.	C 50.11, H 5.84, N 3.25
	Found.	" 50.10, " 5.76, " 3.15

Inosamine-SB Hydrochloride—A 1.2 gm. portion of pure N-acetylinoamine-SB was refluxed with 20 ml. of 6 N hydrochloric acid for 2.5 hours. The residue (1.2 gm.) obtained on vacuum distillation was recrystallized from 30 ml. of water-ethanol-acetone (1:1:1), giving 0.8 gm. of colorless, irregular crystals, which decomposed gradually above 260°.

$C_8H_{14}NO_5Cl$ (215.64).	Calculated, N 6.49; found, N 6.27
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Compounds Derived from dl-epi-Inosose

dl-epi-Inosose Phenylhydrazone—*dl-epi-Inosose* was prepared by the oxidation of inositol with concentrated nitric acid essentially according to the directions of Posternak (3). In larger scale oxidations, it is essential that the residual nitric acid be removed rapidly, either by evaporation in a shallow dish or by use of reduced pressure. Although the inosose can be crystallized from water, a more effective purification is obtained via the phenylhydrazone. Three 80 gm. runs of inositol gave 54 gm. of crude oxidation product which yielded 56 gm. of *dl-epi-inosose phenylhydrazone*

(3), decomposition range 185–195°.⁴ This material was converted to *dl-epi*-inosose. The purified inosose was finally recrystallized from hot water, giving 23.5 gm. (10 per cent based on inositol) of colorless crystals.

The pentaacetate prepared from this material melted at 106–108° in agreement with the reported value for this compound (3).

dl-Inosamine-EA—A 10 gm. portion of *dl-epi*-inosose phenylhydrazone was hydrogenated under the same conditions used for the *scyllo*-phenylhydrazone. The hydrogen uptake reached theoretical in 3 hours. After removal of the catalyst by filtration, the methanolic filtrate was vacuum-distilled. The residue of inosamine and aniline was washed with benzene, giving a granular solid. This material could not be recrystallized and was converted directly to the hexaacetyl derivative.

dl-Hexaacetylinosamine-EA—The above product was refluxed with 140 ml. of acetic anhydride and 1.8 gm. of fused sodium acetate for 1 hour. The clear red solution was vacuum-distilled to dryness. The residue was extracted with chloroform, and the evaporated chloroform extract was recrystallized from 75 ml. of ethanol, giving 7.0 gm. of colorless flat needles (44 per cent yield based on the phenylhydrazone). The crystals melted on the micro block at 160–162°, partially resolidified, and melted again at 184–186°, then again at 192–194°. (In the capillary only the last melting point is observed.) Very little, if any, loss in weight accompanies these changes, and the various melting points may represent polymorphic forms.

$C_{18}H_{28}O_{11}N$ (431.39). Calculated. C 50.11, H 5.84, N 3.24
Found. " 49.84, " 5.47, " 3.38 (Dumas)
" 3.34 (Kjeldahl)

Reacetylation of purified *dl-N*-acetylinosamine-EA gave a hexaacetyl derivative of unchanged melting behavior.

dl-N-Acetylinosamine-EA—Ammonolysis by the procedure described above, followed by concentration and chilling of the methanolic solution, gave a 64 per cent yield of thick colorless needles, melting sharply at 205–206° with decomposition. A sample recrystallized from methanol-benzene for analysis showed no change in melting point.

$C_8H_{18}O_8N$ (221.21). Calculated. C 43.44, H 6.84, N 6.33
Found. " 43.44, " 6.72, " 6.21

dl-Inosamine-EA Hydrochloride—1 gm. of the hexaacetyl derivative was refluxed with 25 ml. of 6 N hydrochloric acid for 30 minutes. The

⁴ From the mother liquors an osazone (decomposition point 182–184°) was isolated by chromatography as previously described (2) for the *scyllo* compound. The osazone gave correct analyses and an ultraviolet absorption spectrum very similar to that of the *scyllo* compound. (*dl-epi*-Inosose could theoretically yield two different racemic osazones.)

residue after vacuum distillation was dissolved in boiling methanol, and the filtered solution concentrated to one-tenth volume. (The product can also be recrystallized from 90 per cent aqueous methanol by adding acetone.) The product (0.4 gm., 80 per cent) separated as colorless crystals melting at 234–236° with decomposition.

$C_6H_{14}O_4NCl$ (215.64). Calculated. C 33.41, H 6.54, N 6.49
Found. " 33.54, " 6.20, " 6.49

The authors wish to acknowledge the assistance of Miss Harriet Neville and Dr. Robert Pogrund in the Van Slyke and Kjeldahl analyses. The remaining analyses were performed by the Clark Microanalytical Laboratories, Urbana.

SUMMARY

1. Three diastereoisomeric inosamines (monoamino analogues of inositol) have been synthesized and characterized as hexaacetyl and N-acetyl derivatives and as hydrochlorides.

2. Reduction of *scyllo*-inosose phenylhydrazone or oxime yields the expected two (*meso*) inosamines, epimeric at the amino group. Each epimer can be obtained in a pure state by taking advantage of the reversed solubilities of the free inosamines and their N-acetyl derivatives.

3. *dl*-*epi*-Inosose phenylhydrazone has thus far yielded only one of the two expected epimeric (*dl*) inosamines.

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THE METABOLISM OF AMERICIUM IN THE RAT*

By KENNETH G. SCOTT, D. HAROLD COPP, DOROTHY J. AXELROD,
AND JOSEPH G. HAMILTON

*(From the Divisions of Radiology, Physiology, Medicine, Medical Physics,
and the Crocker Radiation Laboratory, University of
California, San Francisco and Berkeley)*

PLATES 1 AND 2

(Received for publication, April 29, 1948)

The announcement of the discovery of element 95 was made in 1946 by Seaborg and his associates (1). Like neptunium (element 93), plutonium (element 94), and curium (element 96) this new element, americium, is radioactive and has not been observed in nature in appreciable amounts. During the war an extensive study was made of the metabolic properties of plutonium (2) and several other heavy elements, notably thorium, protoactinium (3), uranium,^{1, 2} and neptunium (3). Similar studies were also made with a large number of the radioactive elements which arise from fission (4). This work was done at the laboratories of the Manhattan Project, located in different parts of the country. Particular attention was directed towards a very detailed investigation of the metabolism of plutonium in rats and other laboratory animals. These studies, initiated at the University of California at Berkeley, were also carried out at the University of Chicago, Oak Ridge, and Los Alamos.

The outstanding characteristic of the metabolism of plutonium is the high degree of localization and prolonged retention of this element in the skeleton following parenteral administration. Unlike calcium, strontium, barium, and radium, which are deposited primarily in the mineral structure of the skeleton, plutonium may be laid down in the uncalcified osteoid matrix of the bone (5). At the present time, it cannot be definitely stated whether or not all of the plutonium so accumulated is in the organic components of the osteoid matrix. A significant fraction of plutonium may be laid down on the adjacent surfaces of the mineral structure of the bone. If this is so, the amount of penetration in the normal adult rat has been demonstrated by the radioautographic techniques to be less than 25 μ . The accumulation of plutonium in the other tissues of the body following parenteral administration is quite small compared to the deposition in the

* This work was performed under contract No. W-7405-eng-48-A of the University of California under the United States Atomic Energy Commission.

¹ Tannenbaum, A., Silverstone, H., and Kozol, J., unpublished data.

² Dowdy, A. H., unpublished data.

skeleton, and, moreover, its elimination from the soft tissues is relatively rapid compared to its extraordinary high degree of retention by the skeleton. Plutonium is not absorbed to any appreciable extent from the digestive tract. In view of these interesting and obviously dangerous metabolic characteristics of plutonium it was felt that a study of the behavior in the body of its recently discovered and immediate chemical neighbor, americium, might prove to be of interest.

Procedures

The isotope of americium employed, Am^{241} , has a half life of 500 years and emits α -particles to form Np^{237} which in turn decays by α -particle emission, with a half life of 2.25 million years, to produce Pa^{233} . The presence of Np^{237} was of no practical concern in the experiments to be described, since its half life is more than 4000 times longer than that of the americium isotope employed, and thus the existence of the neptunium and its radioactive descendents was not detectable by the procedures employed in these studies.

Americium, made available to us by Professor G. T. Seaborg and his associates, was obtained as a solution of AmCl_3 with 15 γ of americium in 1 cc. of 1 N hydrochloric acid. This original solution was diluted with normal saline and carefully brought to pH 5 by the addition of dilute sodium hydroxide. Any further neutralization would have resulted in the precipitation of the highly insoluble americium hydroxide. The final solution contained 0.3 γ of americium per cc., with a radioactivity of 1 microcurie per cc.

Animal Studies—1 cc. of the above solution was administered by intramuscular injection into the left hind leg of each of fifteen adult white rats and the same amount was given by stomach tube to three more animals. In addition, two rats received 5 γ each, by intramuscular injection, for the purpose of preparing radioautographs to study the distribution of this element in bone. The fifteen animals that received the 0.3 γ by intramuscular injection were divided into groups of three and were placed in metabolism cages, which made it possible to collect specimens of both the urine and feces daily. The five groups of rats were sacrificed at 1, 4, 16, 64, and 256 days. The three rats which were given americium by stomach tube were placed in one metabolism cage and daily collections of urine and feces were made. The rats were sacrificed after an interval of 16 days by means of chloroform and the thorax was immediately opened to permit the withdrawal of from 1 to 5 cc. of blood from the heart. The left leg, which was the site of the injection, was removed at the pelvic girdle for separate assay. The remainder of the animal was skinned, and the following organs and tissues were removed and weighed for separate assay: liver, kidney,

spleen, heart, lungs, muscle, bones of the right leg, and the gastrointestinal tract. These tissues, and in addition the left hind leg, excreta, skin, and remaining carcass, were dried for 2 days at 100° and then ashed at from 500–600° for 24 hours. Preliminary tests demonstrated that no measurable amount of the americium was lost by volatilization at the temperature employed for ashing the tissues. The carcass, which was dried and ashed in one piece, was carefully sifted through a fine mesh screen, and the skeleton was thus separated almost completely from the ash of the other remaining tissues of the carcass, which were chiefly muscle, blood, and fat. The rats for radioautographic study of the bone were sacrificed at 16 days.

Method of Assay of Americium in Materials of Biological Origin—The detection and quantitative measurement of α -particles are relatively simple with appropriate equipment. However, owing to the very short range of this type of radiation, it is not possible in most instances simply to place samples of biological materials in the counting device and obtain an accurate determination of the number of disintegrations taking place. The sample must be spread out in a thin and even film whose mass does not exceed 2 to 3 mg. per sq. cm. If this precaution is not observed, a fraction of the emitted α -particles will not be able to enter the detection chamber. Most of the tissues and organs, even after ashing, contain far too much material to make this possible. This problem may be met by separating the radioactive material, in this case americium, from the bulk of the ash of the samples. Since the total dose of americium given was only 0.3 γ , it was necessary to add to the samples a small amount of a non-radioactive element with similar chemical properties to act as a carrier for the tiny traces of americium present. The chemical properties of lanthanum and americium are very much alike in many respects. In these particular experiments, the ashed tissues were dissolved in dilute nitric acid, 2 mg. of lanthanum nitrate were added, and this was followed by the addition of hydrofluoric acid. The americium was carried down quantitatively by the insoluble lanthanum fluoride precipitate. Thus, the americium could be quantitatively separated from several gm. of tissue. The lanthanum fluoride precipitate was then spread out in a thin film on the counting dishes and the americium α -particles measured.

The specific details of this procedure are as follows: (1) The tissue ash was dissolved in 2 N HNO_3 with a concentration of 20 mg. of tissue ash per cc. of acid solution. (2) 2 mg. of $\text{La}(\text{NO}_3)_3$ were stirred into the ash solution used for any particular assay;³ this ranged from 1 to 5 cc.

³ Because of the presence of α -emitting contaminants in some of the c.p. $\text{La}(\text{NO}_3)_3$ available, the latter must be checked for α emitters by running blanks on each batch received. The radioactive contamination present is believed to be actinium, whose chemical properties resemble closely those of lanthanum as well as americium.

of ash solution. (3) The solution in step (2) was made 2.5 N in hydrofluoric acid by the addition of an adequate amount of 8 M HF. (4) The precipitate formed was separated by centrifugation and the supernatant fluid was discarded. (5) The precipitate was mounted on a gold dish by redissolving it in 8 M HNO₃, transferring the solution to the dish, and adding an excess of HF to reprecipitate the lanthanum, which resulted in a thin film upon drying on a hot-plate at a temperature low enough to prevent spattering. (6) The sample was assayed for americium by determining the α -particles emitted by the use of a parallel plate α counter. (7) The mass upon the dish was determined by weighing. A correction for self-absorption was made.

Radioautographic Methods—For the purpose of securing bone radioautographs, thin histological sections, which ranged from 5 to 8 μ in thickness, were prepared from the undecalcified femur by the techniques developed by McLean and Bloom (6) and by Axelrod (7). The relatively large dose of americium employed was necessary in order to secure enough activity in the sections to obtain satisfactory radioautographs. It is believed to be obligatory to avoid decalcification procedures in the preparation of the section for taking the radioautographs so that the possibility of either leaching or migration of the deposited material will be avoided.

Results

The results obtained from assaying the content of americium in the different organs and tissues are expressed as the per cent per organ and per gm. of wet weight. The left hind leg, which was the site of injection, was assayed as a unit. The carcass ash, after separation of the skeleton by the sifting procedure mentioned earlier, was assayed as well as the skeleton itself. The urine and feces from each group of three animals were likewise ashed and assayed and the values obtained divided by 3, which thus gave the average value of excreta for each of the three animals. The total content of americium in all of these organs, tissues, and excreta was obtained by adding up the individual values and this was compared against the amount given. This computation, of course, is a quantitative indication of the recovery of the administered americium and represents the fraction of americium given which could be determined quantitatively by the analytical procedures employed in these studies. The results were fairly satisfactory, because the average recovery value observed in the series of eighteen rats was 87 per cent and the variations in the different groups ranged from 75 to 100 per cent.

The average measured content of americium in different organs, tissues, and the excreta for the intramuscular experiments is shown in Table I.

The amount in the skeleton was obtained by adding twice the measured americium content of the bones of the right hind leg to the value secured from the assay of the major portion of the skeleton that was separated from the carcass ash. This was done in order to correct for the americium content of the bone of the injected left hind leg, which was not included in the rest of the skeleton. The necessity for doing this arose from the fact that from 2 to 40 per cent of the injected americium was retained at the site of administration. Presumably this was due to the precipitation and adsorp-

TABLE I
Observed Distribution of Americium (Am^{241}) Following Intramuscular Injection of 0.8 γ of Solution of $AmCl_3$ into Hind Leg of Rat

	1 day		4 days		16 days		64 days		256 days	
	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.
Lungs	0.13	0.096	0.18	0.11	0.14	0.093	0.11	0.036	0.065	0.017
Heart	0.11	0.12	0.045	0.056	0.083	0.088	0.049	0.044	0.012	0.010
Liver	30.8	3.41	31.0	3.68	10.6	1.13	1.46	0.13	0.53	0.063
Spleen	0.28	0.39	0.083	0.18	0.079	0.11	0.085	0.13	0.045	0.081
Kidney	2.49	1.29	1.97	1.13	1.02	0.52	0.72	0.34	0.27	0.13
Gastrointestinal tract	1.54	0.068	4.06	0.19	1.56	0.075	0.32	0.012	0.12	0.005
Bone	13.1	0.57	16.6	0.57	17.7	0.88	18.9	0.73	19.2	0.73
Skin	1.84	0.056	1.46	0.039	1.47	0.037	0.33	0.008	0.17	0.005
Muscle	0.83	0.006	1.60	0.014	2.06	0.018	1.53	0.012	0.94	0.008
Blood	0.57	0.053	0.12	0.008	0.10	0.007	0.068	0.004	0.01	0.001
Balance	3.68		2.51		1.61		1.59		1.13	
Unabsorbed in injected leg	43.5		12.9		5.37		5.13		1.45	
Urine	3.12		2.80		2.34		4.27		4.67	
Feces	2.66		27.3		32.1		40.6		51.6	
Recovery of injected dose, %	103.2		100.9		74.1		80.6		79.3	

tion of insoluble compounds of americium at the injection site. It is of interest to note that most of the americium remaining behind in the injected leg was found to be in the soft tissue, although the content in the bones of that leg was of the order of twice that of the opposite injected leg. It was not determined whether this was the result of diffusion of the injected solution to the bone surfaces followed by adsorption or whether it occurred during the ashing procedure. The relatively small excess of americium in the carcass ash over the estimated value derived from the measured samples of blood and muscle may be due to the presence of small fragments of

carcass skeleton which passed through the screen employed for sifting out the bones. The estimated americium content of muscle and blood was made on the basis of the values per gm. noted for these two structures and assuming that their total weights in the intact animal were 45 and 6 per cent respectively of the total body weight. In Table II the data are presented after correction for the unabsorbed fraction in the injected leg and for the deviation of the measured recovery value from 100 per cent. The rate of excretion of americium is shown in Text-fig. 1 for both urine and feces. The relative proportion of americium appearing in the liver and

TABLE II

Distribution of Americium (Am^{241}) in Rat Following Intramuscular Injection

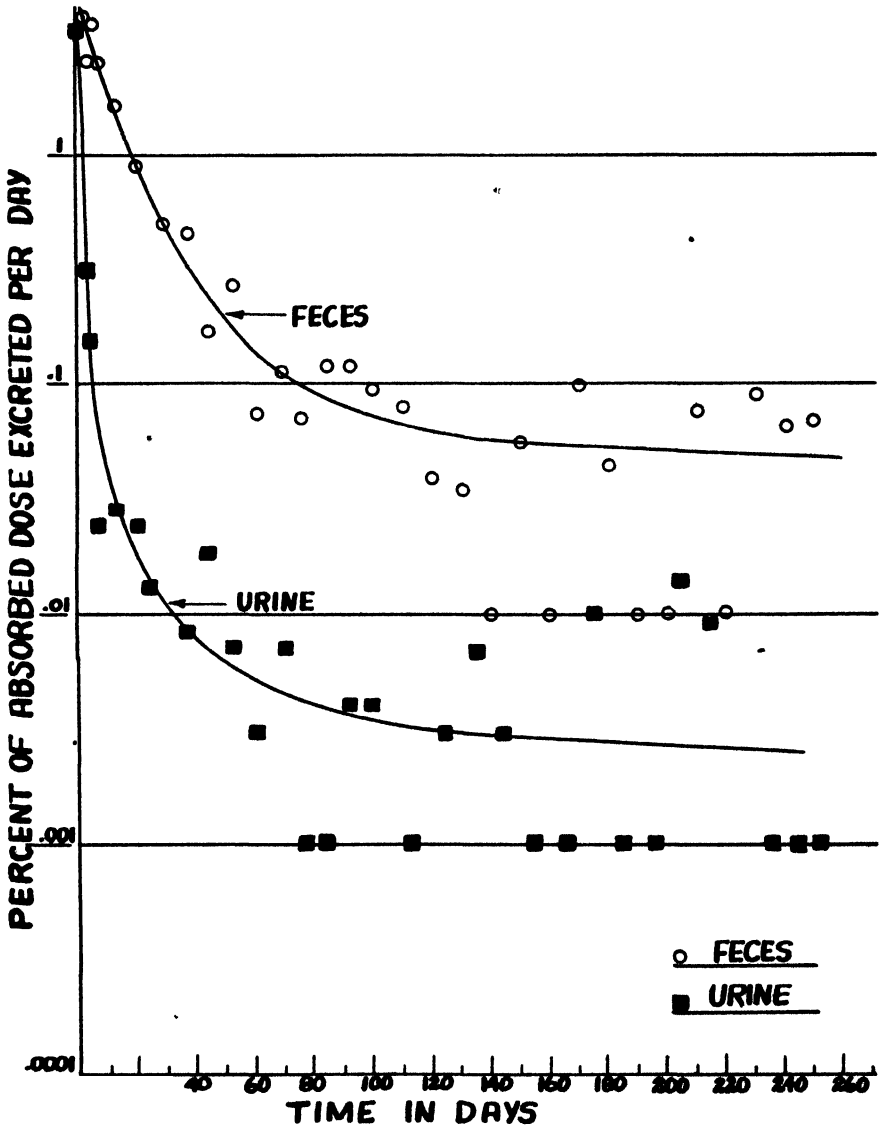
The data, taken from Table I, have been corrected for the fraction of americium unabsorbed at the site of injection and the deviation of the recovery values from 100 per cent.

	1 day		4 days		16 days		64 days		256 days	
	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.	per cent per organ	per cent per gm.
Lungs	0.23	0.17	0.21	0.13	0.21	0.13	0.15	0.048	0.084	0.022
Heart	0.19	0.21	0.052	0.065	0.12	0.13	0.065	0.058	0.015	0.013
Liver	54.8	6.07	35.7	4.24	15.3	1.64	1.94	0.18	0.68	0.081
Spleen	0.50	0.70	0.095	0.21	0.11	0.15	0.11	0.17	0.058	0.11
Kidney	4.43	2.29	2.27	1.30	1.47	0.75	0.96	0.44	0.34	0.16
Gastrointes- tinal tract	2.73	0.12	4.66	0.22	2.25	0.11	0.43	0.016	0.15	0.008
Bone	23.2	1.01	19.1	0.66	25.6	1.25	25.0	0.96	24.8	0.94
Skin	3.26	0.10	1.68	0.045	2.12	0.053	0.44	0.011	0.22	0.006
Muscle	1.46	0.010	1.84	0.017	2.97	0.025	2.03	0.016	1.21	0.010
Blood	1.01	0.095	0.14	0.009	0.16	0.010	0.090	0.005	<0.01	<0.001
Urine	5.53		3.21		3.38		5.66		6.02	
Feces	4.73		31.5		46.4		63.2		66.4	

feces is graphically indicated in Text-fig. 2 and the change of total americium content in the liver and skeleton appears in Text-fig. 3.

The observed and the corrected data for the intramuscular series, shown in Tables I and II, present an interesting finding, namely the high degree of accumulation in the liver and bone. In the case of the liver, most of the accumulated americium is removed at a fairly rapid rate, probably by way of the bile, since the quantity of americium in the feces parallels that lost by the liver; this reciprocal relationship is indicated in Text-fig. 2. After most of the americium is eliminated from the liver, it can be seen in Text-fig. 1 that the excretion is very slow but that the digestive tract still acts as the principal channel of elimination. During the entire period of

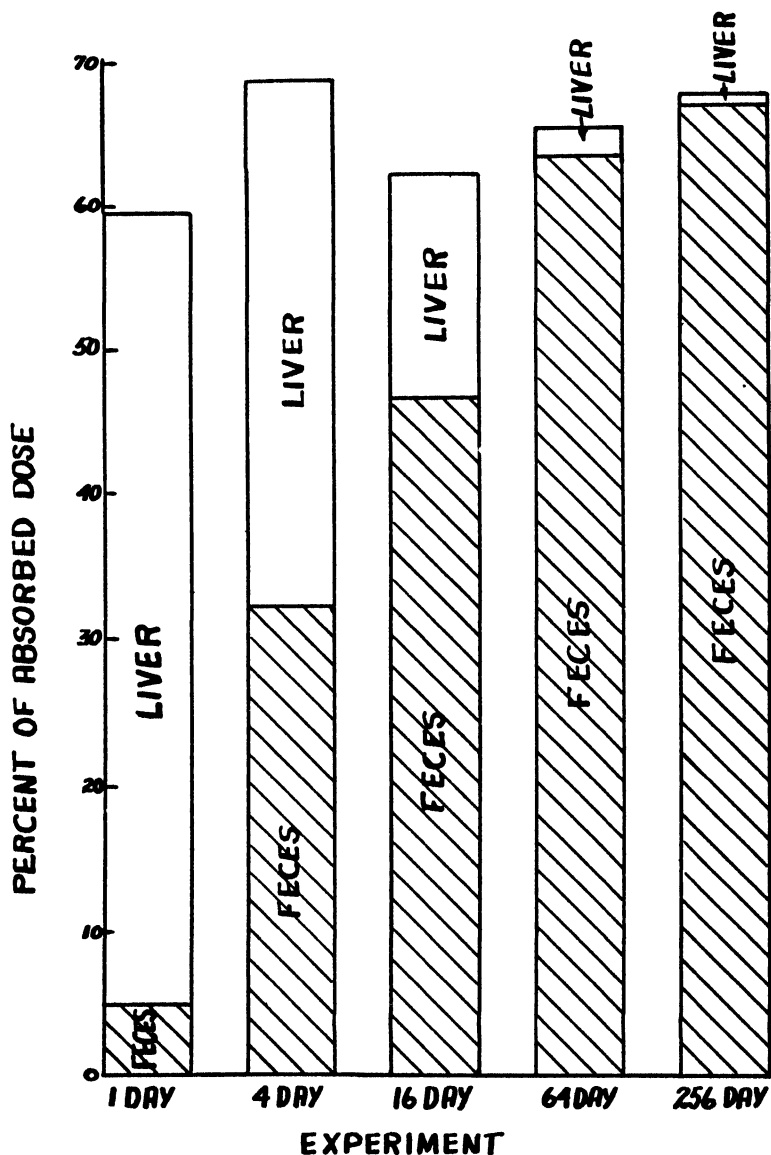
these experiments, including the 256 day interval, there was no significant decrease in the amount of americium in the skeleton. A comparison of the



TEXT-FIG. 1. Fecal and urinary elimination of americium in rats

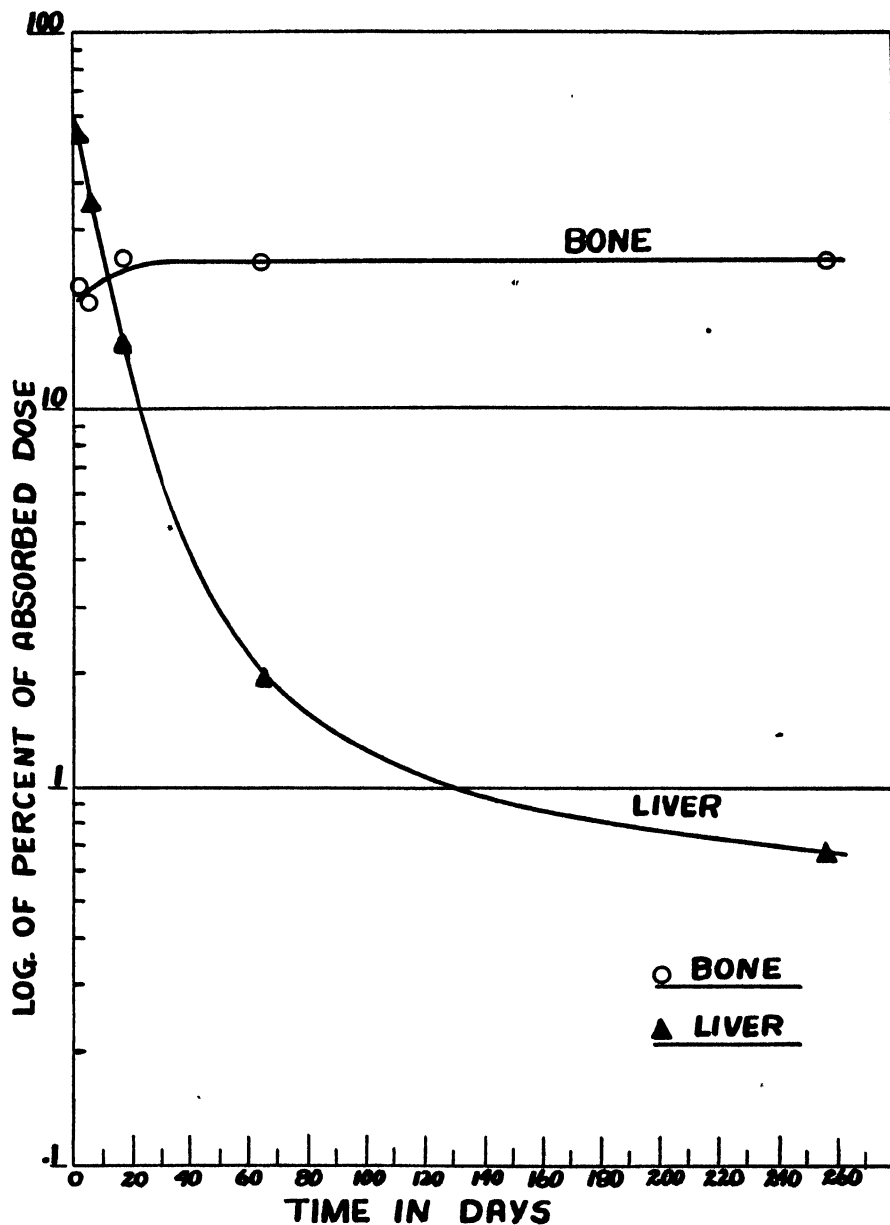
uptake and retention of americium by the liver and bone is given in Text-fig. 3. The only other soft tissues that show significant accumulation are

the spleen and kidneys, whose initial content on a per gm. basis at the earlier time intervals is considerably lower than that of the liver. The fraction of



TEXT-FIG. 2. Deposition of americium in the feces and liver of rats

americium absorbed from the digestive tract following oral administration was observed to be less than 0.01 per cent of the 0.3 γ dose given.



TEXT-FIG. 3. Deposition of americium in the bone and liver of the rat

Radioautographs were made of thin undecalcified sections of the femur to show the actual distribution of the americium in bone. An example is

shown in Fig. 1. It will be noted that the americium appears to be heavily deposited about the trabeculae in the region below the epiphysis and at the endosteal and periosteal surfaces of the shaft, and is rather spottily distributed throughout the cortical bone of the shaft. A higher magnification of the cortical bone (Fig. 2) shows that the spotty intracortical americium corresponds in general location to the small blood vessels of the cortex. Unfortunately, the resolution in the radioautographs is not sufficient to determine whether this material is in the blood vessel walls or in the surrounding mineral structure of the bone. Further technical progress in radioautography is necessary before this problem can be settled.

DISCUSSION

In studies comparable to the series of experiments described here, it has been shown with certain of the fission products in the carrier-free state, as well as with several members of the heaviest elements (4), that the high degree of accumulation and prolonged retention by the skeleton is a phenomenon common to a number of elements. This characteristic of a high degree of accumulation and retention by the skeleton has been demonstrated with the following fission products: yttrium, lanthanum, cerium, praseodymium, element 61, zirconium, columbium, strontium, and barium (4). The heaviest elements that also are deposited in the skeleton include thorium, protoactinium, uranium, neptunium, and plutonium (2-4).^{1, 2} Incomplete data indicate that the same behavior is characteristic of curium (4).⁴ Strontium has been observed to be distributed primarily in the mineral structure of the bone (5), and it is presumed that the same histological pattern of deposition in bone takes place with barium. Radioautographic studies of bone have been made with yttrium, cerium, element 61,⁴ zirconium, strontium, thorium, and plutonium (4, 5). With the exception of strontium, and presumably barium, all of these elements tend to be laid down in the vicinity of the periosteum and endosteum. Heavy deposits occur in the trabecular region, and it is believed that the accumulated radioelements may be largely laid down on the surfaces of the trabeculae. It has already been shown, in the case of americium, that in addition to this site of localization there is apparently some accumulation about the small blood vessels of the cortical bone. This interesting characteristic has been thus far noted only with cerium and element 61. These two rare earth elements show the same very high degree of localization in the liver that has been demonstrated with americium. It is predictable that this radioautographic pattern of the bone, which is characteristic for these three elements, should be observed with lanthanum and praseodymium.

⁴ Scott, K. G., Hamilton, J. G., and Axelrod, D. J., unpublished data.

These two rare earths also demonstrate a similar high level of accumulation by the liver. However, their half lives are too short to permit the preparation of satisfactory bone radioautographs.

The remaining elements subjected to both tracer and radioautographic studies, notably yttrium, zirconium, thorium, and plutonium, exhibit a relatively small uptake by the liver and do not show any significant amount of deposition in the region of the small blood vessels of the cortical bone. The correlation between the high liver uptake and deposition of material about the small blood vessels of cortical bone is a curious phenomenon for which no obvious explanation is available. It is of interest to note that the removal of lanthanum, cerium, praseodymium, and element 61 from the liver proceeds at almost the same rate that has been observed with americium.

It would appear that in the case of americium, as well as with lanthanum, cerium, praseodymium, and element 61, there is a competition between the liver and the skeleton for an accumulation of these elements. Moreover, it is unlikely that any significant fraction of the very constant level of americium in the skeleton is due to transfer to this element from the liver to the bone. Evidence that may be cited for this deduction is based on the fact that by the 64th and 256th days there is no appreciable loss from the skeleton, although by 64 days the americium content of the liver has fallen to a relatively low value. It would be of interest to explore this hypothesis further by performing tracer studies with americium upon hepatectomized animals. With the liver removed, one might expect from 60 to 80 per cent of the americium absorbed from the injection site to be deposited in the skeleton, as is the case for zirconium, yttrium, thorium, neptunium, and plutonium. The reasonableness of this concept is the fact that these elements of somewhat similar metabolic properties, except for their limited deposition in the liver, show an accumulation by the skeleton of the order of 65 to 70 per cent. The possibility that americium and the other substances for which the liver shows such a high affinity are entrapped by the reticulo-endothelial system appears highly unlikely, since the concentration per gm. in the spleen in the earlier phases of these experiments is roughly one-fiftieth of the concentration in the liver.

The practical aspects, with respect to the dangers that might be encountered by those who may work with americium, are fairly obvious. Since it is not absorbed to any significant degree from the digestive tract, the hazard from ingesting this material by mouth is relatively small. The property of selective localization and prolonged retention in bone makes it a highly dangerous agent should it gain entry into the body through cuts, abrasions, etc. In the light of work with other elements showing a close metabolic similarity, it seems probable that soluble compounds of this

element will be absorbed through the alveoli of the lungs.⁵ On the basis of experience with plutonium and mixtures of the fission products, it is likely that if any soluble compound of this element should gain entry into the lungs by inhalation from 10 to 30 per cent might be absorbed into the blood stream and roughly a fifth of this amount could eventually find its way to the skeleton, where it has already been demonstrated there is an extraordinarily high degree of retention.

SUMMARY

An investigation of the metabolism of americium in the rat has been conducted, following its administration by intramuscular injection and by stomach tube. No appreciable absorption took place from the digestive tract. Within 24 hours after parenteral administration, 55 per cent of the total amount of americium absorbed from the site of injection was deposited in the liver and 20 per cent accumulated by the skeleton. Excretion occurred primarily by way of the digestive tract and most of the americium eliminated appeared to come from the liver. There was no significant change in the content of americium in the skeleton throughout the interval of the experiment. The distribution of americium in bone was studied by the radioautographic technique with thin, undecalcified sections of the femur. Americium was heavily deposited in the region of trabecular bone below the epiphysis, at the endosteal and periosteal surfaces of the shaft, and throughout the cortical bone in the region of small blood vessels

We are indebted to Edith Steinhaff for technical assistance.

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⁵ Scott, K. G., Axelrod, D. J., Crowley, J., and Hamilton, J. G., unpublished data.

EXPLANATION OF PLATES

PLATE 1

FIG. 1. Undecalcified section (upper) of rat femur and corresponding radioautograph (lower) showing the deposition of americium in bone. This element is heavily deposited in the region of the trabeculae just below the epiphysis, the endosteal and periosteal surfaces of the shaft, and spottily throughout the cortex. $\times 7$.

PLATE 2

FIG. 2. Higher magnification of bone section and radioautograph shown in Fig. 1. This demonstrates the deposition of americium in the region of the blood vessels in the cortical bone. $\times 232$.



(Scott, Copp, Axelrod, and Hamilton: Americium)



(Scott, Copp, Axelrod, and Hamilton: Americium)

THE DEPOSITION OF URANIUM IN BONE*

I. ANIMAL STUDIES

By W. F. NEUMAN, M. W. NEUMAN, AND B. J. MULRYAN

(From the Division of Pharmacology and Toxicology, Department of Radiation Biology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

(Received for publication, May 3, 1948)

Studies of the distribution of uranium in animal tissues (1, 2) have shown that a large proportion of the injected dose accumulates initially in the bone and kidney. 40 days after injection, however, bone is the only tissue which has retained significant quantities of the metal (2). Because of the importance of bone as a storage site for uranium, more specific information has been obtained concerning the factors which influence the deposition of uranium in bone in the animal.

One of the problems presented by the previous work concerned the differences in the skeletal distribution of uranium which occurred between the sexes. The bones of male rats consistently accumulated greater quantities of uranium than those of females. All of these rats weighed 200 gm. At this weight, the male rats averaged 9 weeks in age, the females 16 weeks. Were these differences related to age or sex?

Recent studies with radioactive phosphorus (3) have emphasized the importance of the relative vascularity of bone in determining the rate and, as a result, the amount of deposition of minerals in bone. This suggested that the differences observed in young male and adult female rats resulted from variation in the relative vascularity of the skeleton with age. To test this hypothesis, normal male and female rats of various ages and rats in a state of severe rickets were injected with uranium and their bones analyzed. An analysis of the content of uranium in different types of bone structures as represented by skull, vertebra, and long bone was also made. Epiphysis, metaphysis, and diaphysis from rabbits, and periosteum from a dog were examined for purposes of comparison. A study of the uptake of radiophosphate in different parts of the skeleton supplemented the experiments with uranium.

EXPERIMENTAL

Methods.

Rats from a colony of Wistar strain animals were maintained on a fox chow diet unless otherwise indicated. After a 3 to 5 day observation

* This paper is based on work performed under contract No. W-7401-eng-49 for the Atomic Energy Project at The University of Rochester.

period, they were injected intraperitoneally with 2.5 mg. of uranium per kilo as a 0.1 per cent aqueous solution of either uranyl nitrate or acetate. This is a toxic and sometimes lethal dose. The rats were sacrificed by decapitation 48 hours after injection. By this time, the distribution of uranium throughout the animal has reached a fairly steady state (2). The bones to be analyzed were removed, freed of flesh, ashed in a muffle furnace, and analyzed by the fluorophotometric method (4).

Analyses of the skull included the jaw and teeth. All femora and humeri of each animal were combined for analysis. Three vertebrae were taken from the lumbar region. In one case, the epiphyses, metaphyses, and shafts of the long bones of two 6 week-old chinchilla rabbits were separated and analyzed.

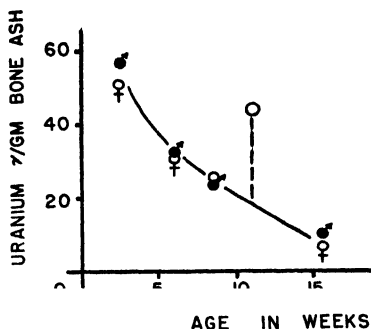


FIG. 1. Effect of age on the deposition of uranium in the femora and humeri of the rat. The curve represents a total of 56 animals, each point being an average of from seven to eleven rats. $\circ$ is the average value for the long bones of each of two rachitic rats.

A similar routine was followed in the investigation of the distribution of radioactive phosphorus. Rats 2 and 15 weeks old were injected intraperitoneally with a saline solution of P^{32} as Na_2HPO_4 , 0.1 mg., 1 microcurie per kilo. After an equilibrating period of 24 hours, the rats were killed, the bones removed, ashed in a muffle furnace, dissolved in HCl , and the radioactivity measurements made in the conventional manner (5).

Results

Concentration of Uranium in Bones at Various Ages—The effect of the age of the rat on the deposition of uranium in bone is clearly demonstrated in Fig. 1. On the basis of ash weight, the young rat (2.5 weeks of age) evidenced a deposition of uranium in the femora and humeri nearly 6 times that which occurred in the adult rat (15 weeks of age). No sex differences were observed over the range of ages studied, including animals before, during, and after sexual maturation.

The results obtained from two 11 week-old rats, which had been maintained for 2 months on a rachitogenic diet,¹ are also recorded in Fig. 1. These animals were in a state of severe rickets. The ash content of their bones was 11 and 15 per cent, respectively, as compared to a value of 40 per cent normally found at this age. The concentration of uranium observed was excessively high if compared with that of normal animals of the same age.

Concentration of Uranium in Bones of Different Structural Types—The relative activity of the skull, vertebra, and combined femur and humerus in accumulating injected uranium and radiophosphate is shown in Table I. In all cases, vertebra fixed greater quantities of uranium than did skull. Long bone was intermediate, but, in general, gave results comparable to

TABLE I
Relative Distribution of Uranium and P³² in Different Bones

Age of animal	Skull		Femur and humerus		Vertebra	
	No. of rats	Uranium	No. of rats	Uranium	No. of rats	Uranium
<i>wks.</i>		γ per gm. ash		γ per gm. ash		γ per gm. ash
2.5	9	26.0	15	54.0	9	64.0
15	4	9.9	4	10.4	3	15.7
		Pm*		Pm*		Pm*
2	12	37.0	12	51.0	12	48.0
15	5	9.9	5	10.7	5	14.2

* To facilitate a comparison with the uranium values the counts per minute per gm. of ash were divided by an appropriate factor.

vertebra in young rats and to skull in adult rats. The differences between the bones were smaller in the adult rats.

The uptake of P³² by the three skeletal components followed a pattern strikingly similar to that seen with uranium.

Material for a comparison of the deposition of uranium in the epiphysis, metaphysis, and shaft of rapidly growing femur was obtained from two young rabbits also given 2.5 mg. of uranium per kilo intraperitoneally.

Analyses of the pooled samples gave the following content of uranium, in micrograms per gm. of ash: metaphysis 2.3, epiphysis 1.8, shaft 1.6.

In this case, the metaphysis accumulated greater quantities per gm. of ash than did the rest of the bone. It is also interesting that smaller quantities of uranium were found in the bones of rabbits than of rats of a corresponding age.

¹ A modified Steenbock diet, Diet 2, obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

Periosteum was obtained from the ribs and long bones of a dog which had received thirteen injections of 0.3 mg. of uranium per kilo (as $\text{UO}_2(\text{NO}_3)_2$) over a period of 6 weeks and a final dose of 5 mg. of uranium per kilo. The dog was sacrificed 9 days after the final injection. For comparison, samples of bone and muscle were taken from each of the areas from which periosteum was obtained. Bone and muscle specimens were pooled separately; periosteum from tibia and femur was kept separate from the periosteum taken from rib. The analytical results are presented in Table II.

The amount of uranium found in muscle is comparable to values reported for such tissues from normal untreated rats (2). The values for periosteum, while significantly higher than for muscle, were low in comparison to those of the adjacent bone. Calcified bone is apparently the principal site of deposition, *not* periosteum.

TABLE II

Comparison of Uranium Content of Bone, Periosteum, and Muscle from Dog after Repeated Uranium Injections

Tissue	Uranium content
	γ per gm. wet weight
Bone	2 1
Periosteum (rib)	0.42
" (tibia and femur)	0 41
Muscle	0 04

DISCUSSION

The age of the rat proved to be an important factor in determining the extent of uranium deposition in bone. The young, more rapidly growing animals, irrespective of sex, showed a greater fixation of injected uranium. A comparison of different parts of the femur also showed a direct correlation between growth activity and uranium fixation.

There seems reason to doubt, however, that the deposition of uranium is directly related to the calcification process *per se*. Rather, it appears more likely that some factor closely associated with bone growth, vascularity perhaps, is responsible. For example, in the rachitic animals, large amounts of uranium were found in bones where no net accretion of mineral was taking place. Radiophosphate, the fixation of which has been established to be principally an exchange process not directly associated with growth (3, 6), showed the same relative distribution as did uranium. Finally, it has been shown (2) that uranium is cleared from the blood within 45 minutes after injection. During this time, the amount of new calcifi-

cation is so small that, to account for uranium deposition as part of the calcification process, nearly every mole of hydroxylapatite laid down must bind a uranyl ion. At the concentrations of uranium in the plasma during this period, such a phenomenon seems improbable.

It has been reported (7) that uranium is deposited principally in the periosteum and endosteum. Direct analyses of periosteum did not confirm this view.

SUMMARY

The deposition of injected uranium in bone was found to be directly related to growth activity. Young rats, irrespective of sex, showed a greater concentration of uranium in bone than did older animals.

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THE DEPOSITION OF URANIUM IN BONE*

II. RADIOAUTOGRAPHIC STUDIES

By M. W. NEUMAN AND W. F. NEUMAN

(From the Division of Pharmacology and Toxicology, Department of Radiation Biology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

PLATES 3 AND 4

(Received for publication, May 3, 1948)

Uranium is deposited in skeletal tissue (1, 2) in concentrations which correspond well with growth activity (3).

A more precise localization of the metal has been made possible by a simultaneous radioautographic and microscopic study of ground sections of bones containing U^{233} , an α emitter of long half life. The results of this study confirmed the correlation between growth activity and uranium uptake. Further, little redistribution from the initial site of deposition was observed over a period of 40 days. These data are in good agreement with the results of an earlier radioautographic study (1).

EXPERIMENTAL

Methods

Four male, Wistar strain rats, weighing approximately 100 gm., were injected intraperitoneally with toxic doses of uranyl acetate (1.5 to 3.0 mg. of uranium per kilo) containing 1.5 microcuries of U^{233} . The animals were sacrificed 4, 48, and 120 hours and 40 days after injection. Bone samples were removed and fixed in chloroform-methanol (1:1). Radioactivity measurements showed that this fixative did not dissolve uranium. Roughly ground sections of the fixed bone were glued to corks and ground again on a marble stone to a thickness of 0.125 to 0.5 mm. The cork-mounted bone sections were then fixed firmly with rubber bands to Eastman α -ray plates for an exposure period of 4 weeks.

Results

Representative autographs with their corresponding bone sections are presented in Figs. 1 to 5.

In confirmation of earlier reports (2), no uranium was found in soft tissue 4 hours after administration. The epiphyseal cartilage of femur (Fig. 3) and the pulp cavity and periodontal membrane of the mandible (Fig. 2)

* This paper is based on work performed under contract No. W-7401-eng-49 for the Atomic Energy Project at The University of Rochester.

were essentially free of activity. In all sections examined, uranium was found only in the calcified portion and here only on surfaces readily available to the circulation; *i.e.*, the surfaces of trabeculae in cancellous bone (Fig. 1), on the inner and outer surfaces of femur shaft, and around large blood vessels and Haversian canals within the compact bone (best seen in Fig. 4).

The most striking concentrations of activity occurred in areas where active calcification was taking place. In the vertebra (cross-section, Fig. 1), which increases in thickness by accretion of bone on its external surfaces, it may be seen that the darkest areas in the autographs occurred at the outer circumference and the external surfaces of the trabeculae. Also corresponding with the direction of growth (4), the greatest concentrations of activity in the mandible (Fig. 2) were found on the crests and distal surfaces of the alveoli. The high activity on the pulp surface of the dentin coincided with the surface on which continuous deposition of mineral occurs. In long bone, calcification occurs in the metaphysis, under the periosteum, and under the articular cartilage of the epiphysis. Again, as shown in Figs. 3, 4, and 5, the greatest concentrations of uranium were found in these areas.

A comparison of the three femurs in Figs. 3, 4, and 5 shows that, once deposited, uranium was virtually fixed. Little redistribution occurred as the growth of bone continued. The increasing width of the space between epiphysis and metaphysis which occurred in the autographs with increasing time after injection reflects the relative displacement of the bone which was deposited at the time of injection. It is interesting that some of the metaphyseal bone containing uranium remained unabsorbed after 40 days, although bone which had been laid down subsequently had already been resorbed. This unresorbed metaphysis is not readily apparent in the photomicrograph, but was easily observed on microscopic inspection. When the 40 day autograph was superimposed on its femur section, it was observed that, at the proximal end of the shaft, bone had been deposited externally to the periosteal line of uranium deposition but had failed to resorb at the marrow surface. This accounts for the excessive thickness of the shaft walls in this area.

DISCUSSION

The fact that the uncalcified tissues of these specimens contained no activity is in agreement with a previous distribution study (2) which showed that as early as 2.5 hours after injection all soft tissues except kidney were practically uranium-free. Hamilton (5) suggests that uranium is deposited in the soft tissue covering the bone surface rather than in the mineral itself. These observations do not confirm his view. The periodontal membrane (Fig. 2), a modified periosteal tissue, contained little or no activity. The

periosteal line of uranium deposition in the 40 day femur was embedded in the middle of the shaft by subsequent accretion of bone. If the activity had been in the periosteum, it would have remained on the outer surface as growth continued. Further, the amount of uranium found in periosteal tissue by direct chemical analysis was low relative to that in the adjacent bone (3).

The concentration of uranium on mineral surfaces adjacent to the circulation and in areas of calcification is in keeping with the results of previous work (2) which showed a correlation between the relative circulation and growth activity of the bones and their ability to fix uranium *in vivo*. It has been suggested, however, that uranium is not precipitated along with the apatite crystals but rather exchanges with some ion or ions on the available, preformed crystal surfaces (3).

It is interesting that the presence of uranyl ions has been shown (6, 7) to decrease the solubility of tooth enamel. Reduced solubility of uranium-impregnated bone may explain the failure of the resorption processes in the metaphysis and in the shaft at the marrow surface in uranium-injected rats.

SUMMARY

Radioautographs of bone containing U^{233} showed this element to be deposited only in the mineral portions and to be particularly concentrated on surfaces adjacent to the circulation and in areas of calcification. Once fixed, little redistribution of uranium occurred. As the normal growth and calcification processes continued, new bone accumulated over the lines of deposition and the resorption of bone to some extent was inhibited, probably because of the insolubility of uranium-impregnated bone salt.

The authors are pleased to acknowledge the able assistance of Mr. Robert Hay in preparing the photographs.

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EXPLANATION OF PLATES

PLATE 3

FIGS. 1 AND 1, A. Photomicrograph and radioautograph of vertebra. Cross-section of a lumbar vertebra removed 2 days after the injection of U^{233} .

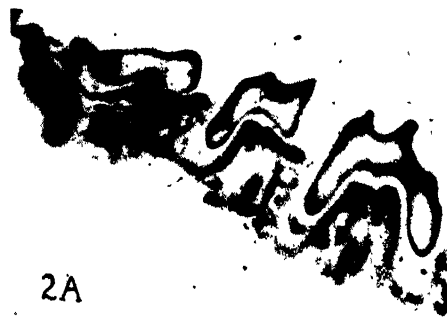
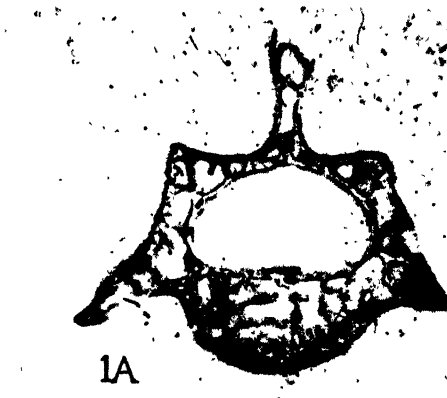
FIGS. 2 AND 2, A. Photomicrograph and radioautograph of mandibular molars. Sagittal section of mandible including third to first molars (left to right), taken 4 hours after the injection of U^{233} . Figs. 1 and 2 have had equivalent magnification.

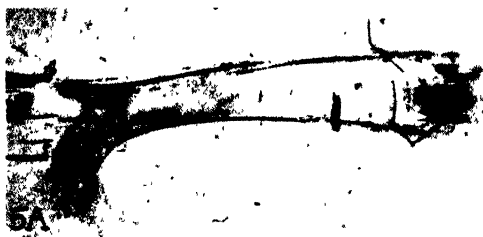
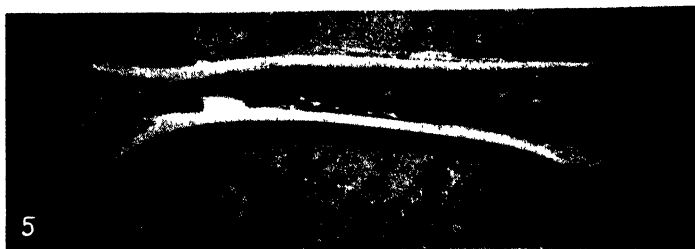
PLATE 4

FIGS. 3 AND 3, A. Photomicrograph and radioautograph of femur. Longitudinal section of distal end of femur taken 4 hours after the injection of U^{233} .

FIGS. 4 AND 4, A. Photomicrograph and radioautograph of femur. Longitudinal section of femur taken 5 days after injection.

FIGS. 5 AND 5, A. Photomicrograph and radioautograph of femur. Longitudinal section of femur taken 40 days after injection. All femur sections have had equivalent magnification.





THE DEPOSITION OF URANIUM IN BONE*

III. THE EFFECT OF DIET

By W. F. NEUMAN, M. W. NEUMAN, EDNA R. MAIN, AND B. J. MULRYAN

(From the Division of Pharmacology and Toxicology, Department of Radiation Biology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

(Received for publication, May 3, 1948)

Studies of the distribution and excretion of injected uranium (1-3) have demonstrated that a considerable proportion of the element is deposited in the skeleton. Mobilization of this fixed uranium was found to be a slow process (2). This was confirmed by radioautographic studies (4) which demonstrated that, once deposited, very little redistribution of the uranium took place.

Since variations in diet have been shown (5) to exert a profound effect in plumbism, it seemed possible that the mobilization of skeletal uranium, too, might be enhanced by proper choice of diet. In support of this view was the finding that alkali administration reduced the quantity of uranium deposited in the kidney (2).

In these experiments, rats, given several injections of uranyl nitrate, were divided into groups and placed on different experimental diets for a period of 50 days. A rachitogenic diet was found to increase the rate of removal of skeletal uranium. Acidic and alkaline diets did not significantly alter the normal, slow mobilization process.

EXPERIMENTAL

Methods

Five female, albino, Wistar strain rats about 3 weeks of age, taken from each of fifteen litters, were divided among five groups. Thus, each group comprised fifteen rats weighing from 45 to 65 gm., with a corresponding litter mate in each of the other four groups.

After a 4 day observation period, a solution of uranium, 0.1 per cent $\text{UO}_2(\text{CH}_3\text{COO})_2$, was administered intraperitoneally to all rats according to the following program: Eight doses of 0.15 mg. of uranium per kilo were injected on alternate days. 2 days after the last small dose, one injection of 2 mg. of uranium per kilo was given. The purpose of the preliminary

* This paper is based on work performed under contract No. W-7401-eng-49 for the Atomic Energy Project at The University of Rochester.

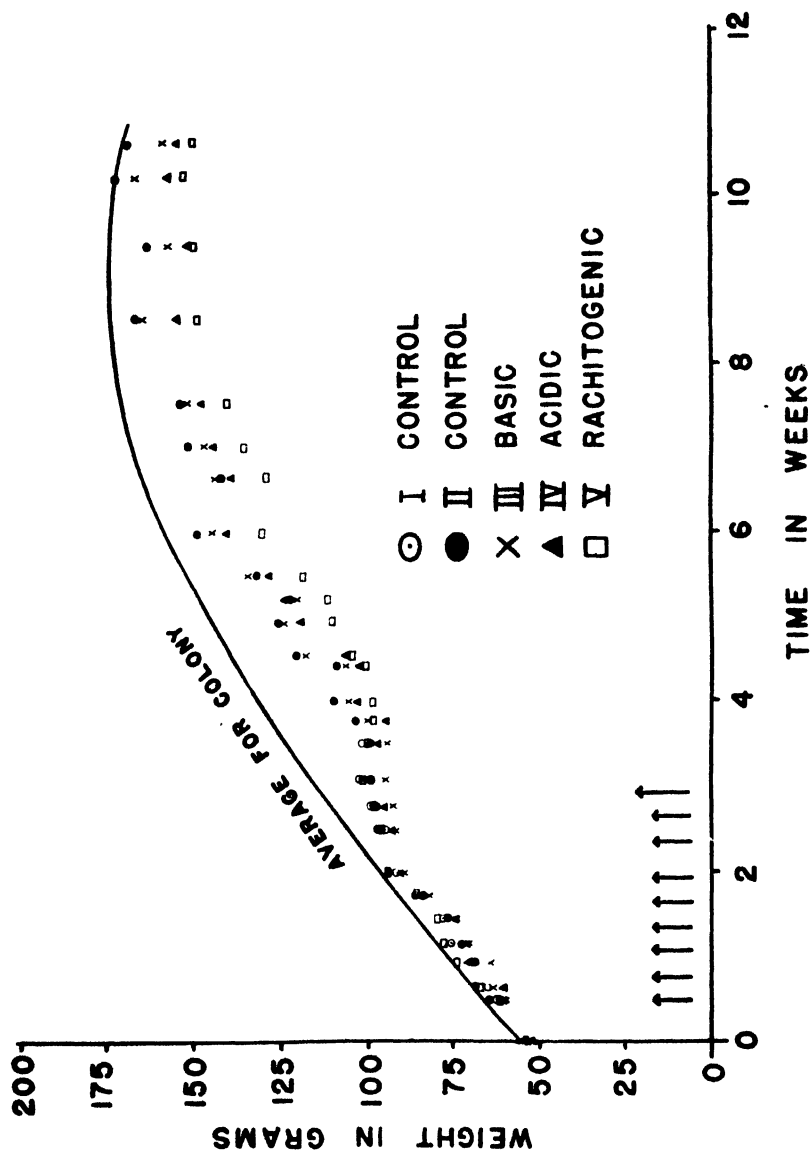


Fig. 1. Effect of uranium injections and experimental diets on growth rate. The arrows indicate injections.

small doses was to establish a tolerance¹ to the large, final dose, which, in untreated rats, is often lethal. In this case, a tolerance developed; only three of 75 rats succumbed. The large dose was necessary in order to obtain skeletal concentrations of uranium adequate for analysis.

3 days after the last injection, Group I was sacrificed for analysis and the other groups were maintained on the following diets for 7 weeks: Group II, a control diet of Purina fox chow, the maintenance diet of all groups through the observation and injection periods; Group III, an alkaline diet, consisting of fox chow supplemented with 0.5 per cent sodium bicarbonate; Group IV, an acidic diet, fox chow supplemented with 0.32 per cent ammonium chloride (the alkaline and acidic diets contained equivalent concentrations of acid and base which were found to be the highest possible

TABLE I

Effect of Experimental Diets on Composition of Pooled Femora and Humeri

The results are expressed in mean per cent plus or minus the standard deviation obtained according to the method of Fisher (7).

Group No.*	No. of animals	Bone composition		
		Water	Organic material†	Ash
I. Control	15	49.3 ± 2.5	21.0 ± 1.3	29.1 ± 1.6
II. Control diet	13	36.6 ± 3.4	23.9 ± 1.8	39.5 ± 3.0
III. Basic diet	15	36.4 ± 2.1	24.1 ± 1.9	39.5 ± 2.4
IV. Acidic diet	14	35.8 ± 2.5	24.2 ± 1.0	40.0 ± 2.2
V. Rachitogenic diet	14	42.8 ± 3.6	25.0 ± 0.8	32.0 ± 3.1
P values, Group II vs. V		<0.01	0.04	<0.01

* Group I, 6 to 7 weeks old. Groups II to V were 13 to 14 weeks old at sacrifice.

† Determined by difference.

concentrations consistent with sufficient dietary intake); Group V, a rachitogenic diet.² Water and food were given *ad libitum*.

The rats were weighed on alternate days for the first 2 weeks of the dietary period and twice a week thereafter.

53 days after the last injection of uranium, Groups II to V were sacrificed by decapitation. The femora and humeri from each animal were removed and scraped free of adhering flesh. Fresh, dry, and ash weights were obtained. The uranium content of each sample of ash was determined by the fluorophotometric method (6).

¹ Haven, F., unpublished results.

² A modified Steenbock diet, Diet 2, obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

Results

The average growth curve of all the experimental animals is compared in Fig. 1 with the growth curve for the average female rat of this colony. The growth rate was retarded somewhat by the administration of uranium, particularly in the case of the rachitic animals.

The effect of the diets on the composition of the long bones is presented in Table I. In spite of the relatively large quantities of alkali (Group III) and acid (Group IV) consumed, no changes were observed in the composition of the long bones. The bones of the rachitic animals, however, showed marked changes as expected; *i.e.*, increased water content and decreased ash content.

To determine the amounts of uranium removed from the bones as a result of the various 7 week dietary régimes, the total uranium content of the pooled femora and humeri of each animal was compared with the average

TABLE II
Effect of Diet on Mobilization of Uranium from Pooled Femora and Humeri

Group No.	No. of animals	Total uranium content	Uranium concentration	Per cent of deposited uranium remaining after 50 days
		γ	γ per gm. ash	
I. Control	15	$12.4 \pm 1.4^*$	40.8 ± 5.7	
II. Control diet	13	7.3 ± 1.9	12.0 ± 1.3	59 ± 11
III. Basic diet	15	7.2 ± 1.3	12.6 ± 2.6	58 ± 11
IV. Acidic diet	14	7.6 ± 1.5	13.1 ± 2.2	61 ± 12
V. Rachitogenic diet	14	4.9 ± 1.5	11.8 ± 3.1	39 ± 12

* Error recorded as standard deviation

total uranium content found in the femora and humeri of Group I, the control animals killed at the beginning of the dietary period. These data are presented in Table II.

Only the rachitogenic diet significantly ($P = <0.01$) increased the removal of uranium from the bone.

DISCUSSION

The mechanism of the action of the rachitogenic diet in increasing the rate of removal of uranium from bone seems clear. It has been shown that the deposition in bone of injected uranium is complete within a few hours (2). Subsequently, in the rapidly growing animals, new bone is deposited over the fixed uranium, thus decreasing the availability of uranium to the circulating fluids. Very little redistribution of uranium to the newly formed bone occurs (4). The rachitogenic diet, then, by lowering the net

accumulation of bone salt, leaves the deposited uranium more available to the circulating fluids (8) and increases the rate of its removal.

At the levels of acid and base administration employed (approximately 6 mm per kilo per day), no alteration in the rate of removal of uranium from the skeleton was observed. A study *in vitro* of the equilibration of bone ash with uranyl bicarbonate solutions³ has shown that high concentrations of bicarbonate inhibit the uptake of uranium by the ash. It would be expected, then, that alkali administration might increase the rate of removal of uranium *in vivo*. Apparently, it is not possible to alter sufficiently the composition of the body fluids to affect an increased mobilization of uranium by dietary supplements of alkali.

It is interesting that nearly one-half of the uranium originally deposited has disappeared in the 7 week dietary period. If the femora and humeri are assumed to be representative of the skeleton as a whole, the half life of skeletal uranium in the rat must be of the order of 50 to 60 days. This would correspond, on a logarithmic basis, to an excretion rate of slightly over 2 per cent per day.

SUMMARY

The effect of several experimental diets on the rate of mobilization of skeletal uranium in rats was tested. Alkaline and acidic diets were without effect; a rachitogenic diet increased the rate of mobilization.

The half life of skeletal uranium in the rat was found to be of the order of 50 to 60 days.

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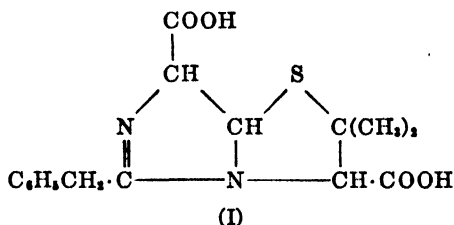
STUDIES ON CRYSTALLINE DL-BENZYLPENICILLINIC ACID

By ARTHUR H. LIVERMORE, FREDERICK H. CARPENTER, ROBERT W. HOLLEY, AND VINCENT DU VIGNEAUD

(From the Department of Biochemistry, Cornell University Medical College, New York City)

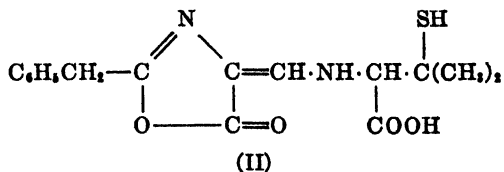
(Received for publication, May 4, 1946)

In a recent communication from this laboratory it was reported that a high yield of D-benzylpenillic acid (I) could be obtained through the con-



densation of D-penicillamine hydrochloride with 2-benzyl-4-methoxymethylene-5(4)-oxazolone and subsequent treatment of the crude condensation product (1). This synthetic D-benzylpenillic acid was identical with that obtained from benzylpenicillin by rearrangement in aqueous solution at pH 2.

The ultraviolet absorption of the crude product from the condensation of D-penicillamine hydrochloride and 2-benzyl-4-methoxymethylene-5(4)-oxazolone in pyridine solution containing triethylamine indicated the presence of a large amount of D-benzylpenicillenic acid, and it was therefore thought that penicillenic acid might be the intermediate compound which yielded penillic acid. Conclusive evidence in favor of this hypothesis has now been obtained through work on DL-benzylpenicillenic acid (II).¹



¹ Methyl D-benzylpenicillenate has been defined (2) as the product obtained upon treatment of benzylpenicillin methyl ester with mercuric chloride. Benzylpenicillenic acid is generally assumed to have structure II. The structure of benzylpenicillenic acid, however, has not been rigorously established (3), nor has D-benzylpenicillenic acid been isolated in crystalline form.

We have now found that when DL-penicillamine hydrochloride is condensed at 75° with 2-benzyl-4-methoxymethylene-5(4)-oxazolone in pyridine solution containing triethylamine a crystalline product can be obtained in high yield. The ultraviolet absorption of this compound showed a maximum at 3225 Å ($E_M = 26,600$)² and the material could be degraded to the sodium salt of 2-benzyl-4-hydroxymethylene-5(4)-oxazolone by treatment with sodium hydroxide (2, 3). These properties were those expected of penicillenic acid (II).

When the crystalline DL-benzylpenicillenic acid was allowed to stand in 95 per cent ethanol solution at room temperature for 24 hours, DL-benzylpenillic acid³ was obtained in 25 per cent yield. These conditions were identical with those which gave D-benzylpenillic acid from the crude condensation product of D-penicillamine and 2-benzyl-4-methoxymethylene-5(4)-oxazolone (1). It can therefore be concluded that D-benzylpenicillenic acid is an intermediate in this synthesis of D-benzylpenillic acid.

It has already been shown (4) that the condensation of D-penicillamine hydrochloride with 2-benzyl-4-methoxymethylene-5(4)-oxazolone produces a small amount of antibiotic activity. This activity is due to the synthesis of a minute amount of benzylpenicillin (5). The DL isomer yields approximately half of the antibiotic activity obtained from the D isomer (4). With crystalline DL-penicillenic acid at hand, an opportunity was afforded to see whether this crystalline material gave antibiotic activity when it was heated in pyridine solution in the presence of pyridinium chloride. Penicillenic acid has been suggested repeatedly (4) as the intermediate compound in the synthesis of antibiotic activity from D-penicillamine hydrochloride and the oxazolone, but this has not been established. The crystalline DL-benzylpenicillenic acid was therefore heated in pyridine solution in the presence of pyridinium chloride at 110° for 12 minutes. A small amount of antibiotic activity was obtained (less than 0.1 percent of the theoretical amount). This amount was comparable to that formed when DL-penicillamine hydrochloride and 2-benzyl-4-methoxymethylene 5(4)-oxazolone were condensed under the same conditions.

Because of the small yield of activity, it is difficult to eliminate completely the possibility that the activity is formed from an impurity very closely associated with the penicillenic acid. However, the DL-penicillenic acid was subjected to a series of recrystallizations and the amount of antibiotic activity obtainable from each fraction was determined by heating it with

² E_M is the molar absorption coefficient and is equal to D/cd , where D is $\log I_0/I$, c is the concentration in moles per liter, and d is the cell thickness in centimeters.

³ In a forthcoming communication from this Laboratory, evidence will be presented to show that this racemic benzylpenillic acid contains a D moiety which is identical with the D-benzylpenillic acid obtained on rearrangement of D-benzylpenicillin.

pyridine and pyridinium chloride. These repeated recrystallizations of the DL-penicillenic acid did not result in any loss of its ability to produce antibiotic activity.

EXPERIMENTAL

Preparation of DL-Benzylpenicillenic Acid (II)—1.8 gm. of DL-penicillamine hydrochloride and 1.98 gm. of 2-benzyl-4-methoxymethylene-5(4)-oxazolone were heated in 360 cc. of pyridine and 15 cc. of triethylamine at 75° for 20 minutes. The solution was evaporated *in vacuo* under nitrogen to a gum which was then dissolved in 300 cc. of chloroform. The chloroform solution was washed with 300 cc. of ice-cold phosphate buffer of pH 1.6 (2 M) and then with 300 cc. of cold phosphate buffer of pH 5 (1.25 M). After being dried over sodium sulfate, the chloroform solution was evaporated to a yellow powder, which weighed 2.3 gm. This material was washed with two 5 cc. portions of chloroform, and the pale yellow residue was dissolved in 150 cc. of methyl acetate. The solution was filtered and 200 cc. of hexane were added. The solution deposited 872 mg. of crystals, which had a micro melting point of 136–137°. A solution of this material in 95 per cent ethanol (0.01 mg. per cc.) had a maximum in the ultraviolet absorption spectrum at 3225 Å, $E_M = 25,600$. This material was recrystallized from methyl acetate-hexane. The recrystallized product melted at 136–140° (micro), and an ethanol solution (0.01 mg. per cc.) had an absorption maximum at 3225 Å, $E_M = 26,600$.

$C_{16}H_{18}O_4N_2S$ (334.4). Calculated, N 8.38, S 9.59; found, N 8.19, $\bar{S}$ 9.52

Alkaline Degradation of DL-Benzylpenicillenic Acid—A 100 mg. sample of DL-benzylpenicillenic acid was treated with 0.4 cc. of 3 N NaOH in a procedure similar to that used in the degradation of methyl D-benzylpenicillenate (3). The solution was allowed to stand for 25 minutes at room temperature, and was then cooled in an ice bath. The crude sodium salt of 2-benzyl-4-hydroxymethylene-5(4)-oxazolone which separated was washed with methanol and dried. It weighed 34.8 mg. and was recrystallized from methanol-ethyl acetate to yield 6.7 mg. of crystals in the first crop. This material melted with decomposition at 229–231° (micro) and at 218–220° (capillary). After another recrystallization the compound melted at 228–231° (micro) and at 224–225° (capillary). Admixture of this material with an authentic sample of the sodium salt of 2-benzyl-4-hydroxymethylene-5(4)-oxazolone did not depress its capillary melting point. The ultraviolet absorption likewise corresponded to that given by the authentic sodium salt of 2-benzyl-4-hydroxymethylene-5(4)-oxazolone. The ultraviolet absorption maxima in methanol occurred at 2390 Å ($E_M = 6430$) and at 2980 Å ($E_M = 17,650$).

Formation of DL-Benzylpenillic Acid (I) from DL-Benzylpenicillenic Acid (II)—198 mg. of twice recrystallized DL-benzylpenicillenic acid were dissolved in 6 cc. of 95 per cent ethanol. The solution was allowed to stand for 24 hours at room temperature and 45.6 mg. of needles, micro m.p. 178–180°, were collected. When the filtrate was allowed to stand at 5°, it deposited another 5.1 mg. of needles, micro m.p. 177–178°, making a total yield of 25.6 per cent of the theoretical amount. The material was recrystallized twice from NaOH solution by the addition of dilute HCl.

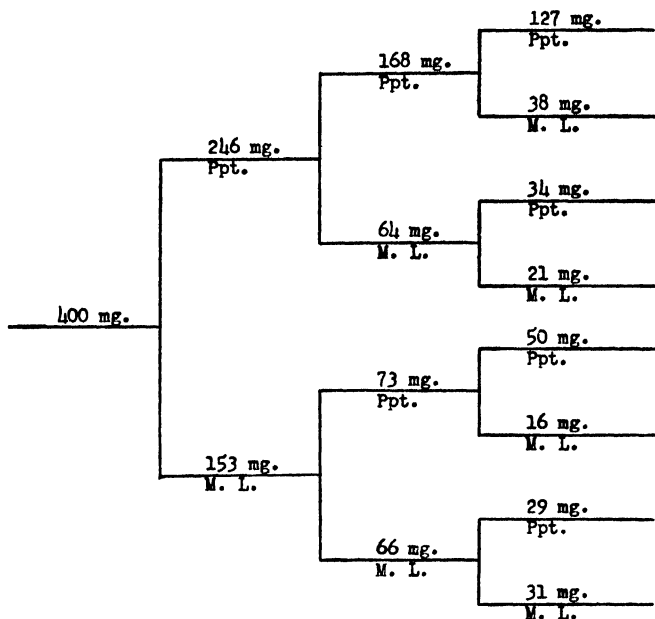


FIG. 1. At each succeeding step, the material was dissolved in methyl acetate and precipitated with hexane (*ppt.*). The mother liquors (*M. L.*) were evaporated and the residues were fractionated in the same manner. All fractions were crystalline with the exception of Fraction G. Aliquots of each fraction were removed for assay and ultraviolet absorption measurements.

The product had a micro melting point of 175–177°. The ultraviolet absorption spectrum of the solution in 95 per cent ethanol (0.01 mg. per cc.) showed a shoulder at 2325 Å, $E_M = 7100$. A similar solution of D-benzylpenillic acid prepared from benzylpenicillin had an almost identical absorption spectrum with a shoulder at 2350 Å, $E_M = 7100$.

$C_{16}H_{18}O_4N_2S$ (334.4). Calculated, N 8.38, S 9.59; found, N 8.04, S 9.37

Formation of Antibiotic Activity from Crystalline DL-Benzylpenicillenic Acid—A 400 mg. sample of crystalline DL-benzylpenicillenic acid was frac-

tionated by successive recrystallizations from methyl acetate-hexane, as illustrated in Fig. 1. An aliquot of each fraction was dissolved in 95 per cent ethanol and the E_M of the ultraviolet absorption maximum at 3225 Å was determined. Also, a 5 mg. aliquot of each fraction was heated at 110° for 15 minutes in 0.5 cc. of pyridine containing 3.12 mg. of pyridinium chloride. The solution was cooled and the pyridine removed *in vacuo*. The residue was dissolved in 5 cc. of 1 per cent phosphate buffer (pH 6) and assayed with *Bacillus subtilis*, ATCC 6051, by a modification of the method of Vincent and Vincent (6) with crystalline sodium benzylpenicillin as the standard.

For each fraction, the ratio of the antibiotic activity produced to the E_M of the absorption maximum at 3225 Å was calculated, as shown in

TABLE I
Properties of Fractions from Recrystallization of DL-Benzylpenicillenic Acid

Fraction	E_M at 3225 Å (a)	Antibiotic activity produced (b)	$\frac{(b)}{(a)} \times 10^4$
		units per mg. of original fraction	
Starting material	26,400	1.24	4.70
A	25,600	1.14	4.45
B	23,000	1.12	4.87
C	25,500	1.26	4.94
D	19,400	1.00	5.15
E	26,600	1.28	4.81
F	21,000	1.04	4.96
G	3,190	<0.25	
H	16,600	0.70	4.22

Table I. These ratios remained quite constant, ranging from 4.22×10^{-4} to 5.15×10^{-4} .

SUMMARY

The preparation of DL-benzylpenicillenic acid in good yield from crystalline DL-benzylpenicillenic acid has established beyond doubt that the latter compound is an intermediate in the synthesis of DL-benzylpenicillenic acid from DL-penicillamine hydrochloride and 2-benzyl-4-methoxymethylene-5(4)-oxazolone. Since the syntheses of the D- and L-penicillenic acids from the D- and L-penicillamine hydrochlorides proceed in an entirely similar fashion, the corresponding penicillenic acids are undoubtedly the intermediates in these cases also.

When crystalline DL-benzylpenicillenic acid was heated in pyridine containing pyridinium chloride, antibiotic activity was obtained. The

yield of activity was comparable to that obtained by condensation of DL-penicillamine hydrochloride and 2-benzyl-4-methoxymethylene-5(4)-oxazolone.

The authors wish to thank Dr. J. R. Rachele and Miss Josephine E. Tietzman for the microanalyses, and Mrs. Mary McKee, Mrs. Elizabeth Mitchell, and Miss Jeannette Treiber for the microbiological assays.

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OBSERVATIONS CONCERNING THE CAUSES OF THE EXCESS EXCRETION OF URIC ACID IN THE DALMATIAN DOG*

BY MEYER FRIEDMAN AND SANFORD O. BYERS

*(From the Harold Brunn Institute for Cardiovascular Research, Mount Zion
Hospital, San Francisco)*

(Received for publication, March 13, 1948)

Ever since Benedict (1) observed that the Dalmatian dog excreted more uric acid than the non-Dalmatian dog, various investigators have attempted to detect some anomaly in the purine metabolism of this species of animal. However, no adequate explanation has been found despite the careful studies of Wells (2) and Klemperer, Trimble, and Hastings (3). Thus the liver of the Dalmatian has been found to contain apparently as much adenase, guanase, and uricase as the liver of the non-Dalmatian (2, 3). As a matter of fact, these investigators concluded that no quantitative relation existed between the uric acid excretion of the Dalmatian and the uricase content of its liver.

It seemed important to reinvestigate this problem. Our approach was to explore three possible causes for the occurrence of excess uric acid in the urine of the Dalmatian; namely, (1) that it was due to an accelerated total purine metabolism, (2) that it was due to a failure of the liver of the Dalmatian to convert uric acid into allantoin, or (3) that it was due to an anomaly in the kidney of the Dalmatian which allowed the more rapid escape of uric acid. The final results of our study indicated that the principal cause of the excess uric acid in the urine of the Dalmatian was the presence of a renal anomaly.

Methods

Three thoroughbred male Dalmatian dogs approximately 1 year of age were used in this study. Two of the dogs (Nos. D1 and D2) were litter mates but the third dog (No. D3) was obtained from another kennel. The dogs were fed on Purina dog chow. Mongrel dogs fed the same food were used for control studies.

All uric acid determinations of either plasma or urine were performed according to the method of Folin (4). Creatinine determinations were done according to the method of Folin and Wu (5), and allantoin in either plasma or urine was analyzed according to the method of Christman, Foster, and Esterer (6) as modified by us (7).

* Aided by grants from the United States Public Health Service and the Wine Institute.

All renal clearances (uric acid, allantoin, and creatinine) were ascertained on anesthetized dogs as described in a previous report (8).

Results

Blood Concentration of Uric Acid and Allantoin—The uric acid content of the blood plasma of three fasting Dalmatians was determined twenty-two times. Nine similar determinations also were made on the plasma of four non-Dalmatian dogs. It was found (see Table I) that the average plasma uric acid concentration in the three Dalmatians was 0.58 mg. per 100 cc. (or 0.20 mg. of uric acid nitrogen per 100 cc.). The average plasma uric acid concentration of the non-Dalmatian dogs was 0.33 mg. per 100 cc. (or 0.11 mg. of uric acid nitrogen per 100 cc.). The average plasma uric acid concentration of the Dalmatian was significantly higher than that of the non-Dalmatian dog.

On the other hand when nine determinations of the plasma allantoin of two Dalmatian dogs were made (see Table I), the average allantoin concentration of 0.61 mg. per 100 cc. (or 0.23 mg. of allantoin nitrogen per 100 cc.) was less than the average (1.01 mg. per 100 cc. or 0.33 mg. of allantoin nitrogen per 100 cc.) of the non-Dalmatian dogs.

Thus the plasma uric acid concentration of the Dalmatian was found to be higher than that of the non-Dalmatian and the plasma allantoin content of the former dog was less than that of the latter. When the average concentrations of total plasma nitrogen (derived from purines) of both groups of dogs were calculated, however, it was found (see Table I) that they were approximately the same (Dalmatian, 0.40 mg. per 100 cc.; non-Dalmatian, 0.44).

Excretion of Uric Acid and Allantoin in Dalmatian and Non-Dalmatian Dog—The average amount of uric acid and allantoin excreted per minute by two Dalmatian dogs (Dog D1, weight 20 kilos; and Dog D2, weight 25 kilos) over a period of 1 hour was determined on nine separate occasions. The same procedure also was carried out nine times on four non-Dalmatian dogs (average weight 20 kilos).

As was expected (see Table I), the average uric acid excretion (0.45 mg. or 0.15 mg. of uric acid nitrogen per minute) of the Dalmatians was approximately 11 times greater than that (0.04 mg. per minute) of the non-Dalmatian dogs. However, the average allantoin excretion of the Dalmatian (0.45 or 0.16 mg. of allantoin N₂ per minute) was about half the amount (0.82 mg.) excreted by non-Dalmatian dogs. The total excretion derived from purines, however, was about equal in the two groups of animals. That is, the average excretion of nitrogen (allantoin and uric acid N₂) was 0.32 mg. per minute in the Dalmatians and 0.30 mg. per minute in the non-Dalmatian dogs (see Table I). In summary then, although the Dalmatian

excreted much more uric acid than the non-Dalmatian dog, its total urinary excretion of allantoin was sufficiently reduced so that its excretion of both purine end-products was neither higher nor lower than that of the other type of dog.

The above results indicated clearly that the excess uric acid found in the urine of the Dalmatian could not be due to an accelerated purine metabolism,

TABLE I

Blood Concentration and Renal Excretion of Uric Acid and Allantoin in Dalmatian and Non-Dalmatian Dogs

Dog No.	Plasma uric acid		Plasma allantoin		Total plasma purine N ₂	Uric acid excreted		Allantoin excreted		Total purine N ₂ excreted
	Total	N ₂	Total	N ₂		Total	N ₂	Total	N ₂	
Dalmatians										
D1	mg. per 100 cc. 0.52 (9)*	mg. per 100 cc. 0.17 (9)	mg. per 100 cc. 0.57 (6)	mg. per 100 cc. 0.22 (6)	mg. per 100 cc. 0.39	mg. per min. 0.41 (6)	mg. per min. 0.14 (6)	mg. per min. 0.44 (6)	mg. per min. 0.16 (6)	mg. per min. 0.30
D2	0.49 (7)	0.17 (7)	0.64 (3)	0.23 (3)	0.40	0.48 (3)	0.16 (3)	0.45 (3)	0.16	0.32
D3	0.74 (6)	0.25 (6)								
Average....	0.58	0.20	0.61	0.23	0.40	0.45	0.15	0.45	0.16	0.31
Non-Dalmatians										
M1	0.35 (1)	0.12 (1)	0.80 (1)	0.28 (2)	0.40	0.03 (1)	0.01 (1)	0.82 (1)	0.29 (1)	0.30
M2	0.40 (2)	0.13 (2)	1.35 (2)	0.48 (2)	0.51	0.05 (2)	0.02 (2)	0.92 (2)	0.32 (2)	0.34
M3	0.25 (5)	0.08 (5)	0.90 (5)	0.32 (5)	0.40	0.03 (5)	0.01 (5)	0.78 (5)	0.28 (5)	0.29
M4	0.30 (1)	0.10				0.03 (1)	0.01 (1)	0.77 (1)	0.27 (1)	0.28
Average.....	0.33	0.11	1.02	0.36	0.44	0.04	0.01	0.82	0.29	0.30

* Numerals in parentheses indicate the number of separate determinations.

since the blood and urine contained approximately the same amount of purine end-products as was found in the blood and urine of non-Dalmatian dogs. Furthermore, the greater concentration of uric acid in the blood of the Dalmatian dog could not explain the excess uric acid in the urine, because, as demonstrated later, artificial elevation of the plasma uric acid of normal dogs to levels 10 times higher than that of the Dalmatian still did

not lead to an excretion of uric acid comparable to that found in the urine of the latter dog.

The evidence obtained suggested that the Dalmatian either was unable to convert sufficient uric acid into allantoin or that it had an *unusual ability to excrete uric acid*.

In any effort to determine a defect in the Dalmatian's ability to convert uric acid into allantoin, it must be stressed that this defect would be found to be a comparative, not an absolute, one, because, as was noted above, about half of the purine is converted and excreted as allantoin. This possible relative rather than absolute deficiency perhaps explains the inability of previous investigators (2, 3) to detect a gross peculiarity in the purine metabolism of this animal.

In order to detect any relative deficiency in the Dalmatian's ability to oxidize uric acid into allantoin, and to eliminate a possible renal factor, it was thought advisable to suppress renal excretion. If this animal were not able to convert uric acid into allantoin adequately, then the uric acid would accumulate in the blood to a much greater extent than allantoin after renal excretion had been abolished.

Accordingly, two Dalmatians (Dogs D1 and D2) and two non-Dalmatian dogs were operated upon after blood samples had been obtained. The ureters of each dog were exposed, then surrounded (at their junction with the renal pelvis) by a thin section of latex rubber tubing (8 mm. in length, 3 mm. in diameter). A silk thread was placed about the rubber tubing and a suture was made, completely compressing the ureter within the tubing. In this manner, the ureters were occluded securely without any danger of cutting them. The bladders of the dogs were then catheterized and any residual urine present was discarded. 24 hours later, second blood samples were obtained and the dogs were reanesthetized and catheterized. The ureters were exposed again and the sutures compressing them were released. During the 24 hour period following ureteral occlusion, none of the dogs urinated nor was any urine found in their bladder at the second catheterization. Moreover, the kidneys of the dogs after occlusion were distended markedly, indicating complete urinary retention. It was estimated that approximately 50 cc. of urine had accumulated in each kidney before the back pressure occasioned by the ureteral occlusion was high enough to effect complete cessation of renal excretion. Within 6 hours following the release of the ureters, all animals began to void urine.

As was expected (9), the non-Dalmatian dogs exhibited no rise in blood uric acid after the 24 hour period of renal shutdown. The plasma uric acid was 0.36 and 0.39 mg. per 100 cc. respectively in the two dogs before and 0.25 and 0.33 mg. per 100 cc. 24 hours after the bilateral ureteral ligation. The plasma allantoin concentration, however, of these two dogs increased

markedly, rising in the 24 hour period from 0.5 and 1.45 to 11.35 and 16.0 mg. per 100 cc., respectively.

Renal suppression in the Dalmatian, however, was found to effect a rise in both uric acid and allantoin. Thus the plasma uric acid and allantoin of Dog D1 were 0.6 and 0.7 mg. per 100 cc., respectively, before, and 3.2 and 5.7 mg. per 100 cc. 24 hours after renal inhibition. Likewise, in Dog D2, the plasma uric acid and allantoin were 0.85 and 0.9 mg. per 100 cc., respectively, before and 2.49 and 13.4 mg. per 100 cc. 24 hours after renal inhibition. These results clearly indicated that, although the Dalmatian dogs had the ability to convert a considerable amount of retained uric acid into allantoin, they nevertheless exhibited a partial or relative deficiency in this regard when compared to the non-Dalmatian dog. Thus, whereas the non-Dalmatians were able to convert all retained uric acid into allantoin, one Dalmatian (Dog D1) appeared to convert only 64 per cent of its retained uric acid and the other (Dog D2) 84 per cent of it.

Renal Clearance (Endogenous and Exogenous) and Allantoin in Dalmatian Dog—Although the anomaly demonstrated above might possibly have explained the higher plasma uric acid of the Dalmatian, it did not explain necessarily the excess uric acid in the urine. Therefore, it was thought desirable to investigate the renal dynamics of this animal.

Twenty-two uric acid and creatinine and nine allantoin clearance studies were carried out on the three Dalmatian dogs. Similarly, ten uric acid, creatinine, and allantoin clearances were performed for control purposes on four non-Dalmatians.

The average endogenous uric acid clearance of the four control dogs was found (see Table II) to be 24.2 cc. per minute or about 26 per cent of the creatinine clearance (92.9 cc.). The average endogenous allantoin clearance (96.0 cc.), as has been described previously (8), was found to be approximately the same as the creatinine clearance. These results demonstrated the fact that the non-Dalmatian dog was not able to excrete uric acid in the same fashion (*i.e.* at the level of glomerular filtration) as allantoin.

However, the Dalmatian dogs were found to excrete endogenous uric acid at the same rate as creatinine, *namely at the level of glomerular filtration*. Thus (see Table II) the average uric acid clearance of Dog D1 was 88.3 cc. per minute and the average creatinine clearance was 92.5 cc. per minute. Similarly in Dog D2, the average uric acid and creatinine clearances were 86.0 and 87.2 cc. per minute, respectively; in Dog D3, they were 76.5 and 74.5 cc. per minute, respectively. The average allantoin clearances of two of the Dalmatians (see Table II) were similar to those of the non-Dalmatians in also being at the level of glomerular filtration.

The above discovery that uric acid probably was a glomerular filtrate without subsequent tubular secretion or reabsorption was confirmed when

studies of uric acid clearance were repeated on both Dalmatian and non-Dalmatian dogs in which the plasma uric acid concentration had been raised by an intravenous infusion of uric acid (500 mg. per 100 cc.) performed 30 minutes before and during the clearance. The results of these studies (see Table III) indicated decisively that uric acid was excreted in the Dalmatians as a glomerular filtrate without subsequent tubular secretion or reabsorption. Thus, despite the fact that the plasma uric acid level of the two Dalmatians was raised many times higher than the endogenous level (in Dog D1, *e.g.*, a plasma uric acid concentration of 44.8 mg. per 100

TABLE II

Uric Acid, Allantoin, and Creatinine Clearance in Dalmation and Non-Dalmatian Dogs

Dog. No.	No of clearances	Average urine volume	Average uric acid clearance	Average allantoin clearance	Average creatinine clearance	Ratio, uric acid-allantoin clearance	Ratio, uric acid-creatinine clearance
Dalmatians							
D1	9	5.43	88.3	85.8	92.5	1.03	0.95
D2	7	5.33	86.0	86.2	87.2	1.00	1.02
D3	6	2.83	76.5		74.5		1.03
Average.....		4.53	83.6	86.0	84.7	1.02	1.00
Non-Dalmatians							
M1	2	4.05	10.75	89.5	85.5	0.12	0.13
M2	2	4.90	28.00	93.5	92.0	0.30	0.30
M3	5	5.60	30.50	105.0	107.0	0.29	0.29
M4	1	6.20	27.50		87.2		0.32
Average.....		5.19	24.2	96.0	92.9	0.24	0.26

All clearances are corrected to 1 sq.m. of surface area.

cc. was obtained during one clearance), the uric acid clearance neither exceeded nor fell below the creatinine clearance concomitantly obtained.

In the non-Dalmatian dogs, however, (see Table III) despite the elevation of plasma uric acid obtained, the clearance of uric acid never equaled the creatinine clearance. Moreover it was observed that, even when the plasma level of uric acid of these latter dogs had been raised to a value 10 times that of the endogenous level of Dalmatian dogs, the amount of uric acid excreted per minute was still less than that excreted by the Dalmatian dog. This last observation demonstrated that the cause of the excess uric

acid in the latter dog's urine was not the result of its somewhat higher plasma uric acid content.

TABLE III

Uric Acid Clearance of Dalmatian and Non-Dalmatian Dog at High Plasma Levels of Uric Acid

Dog No.	Plasma uric acid	Urine volume	Uric acid clearance	Creatinine clearance	Ratio, uric acid-creatinine clearance
Dalmatians					
D1	mg. per 100 cc.	cc. per min.	cc. per min.	cc. per min.	
	10.2	3.92	70.0	71.5	0.98
"	12.2	4.70	93.8	89.6	1.04
"	14.3	7.10	79.7	90.0	0.89
"	15.8	4.82	86.4	83.3	1.04
"	18.2	7.65	98.0	93.0	1.05
"	24.1	4.10	98.0	98.0	1.00
"	40.1	3.20	90.0	93.0	0.97
"	44.8	3.50	98.0	95.8	1.02
Average ...		4.87	89.2	89.3	1.00
D2	7.93	6.60	100.0	93.8	1.06
"	8.23	5.70	103.0	102.0	1.01
"	8.90	3.60	99.0	100.0	0.99
"	10.40	3.00	92.0	96.5	0.95
"	12.20	5.10	90.0	83.0	1.08
"	13.00	4.20	75.7	68.5	1.10
Average....		4.70	93.3	90.6	1.03
Non-Dalmatians					
M1	4.80	5.40	37.0	91.0	0.41
M2	10.25	5.00	39.0	69.5	0.56
M2	13.40	4.06	28.3	86.5	0.33
M3	19.30	4.70	43.0	73.5	0.59
M4	23.50	5.80	44.4	79.0	0.56
Average....		4.99	38.3	79.9	0.49

All clearances are corrected to 1 sq.m. of surface area.

DISCUSSION

The results of the foregoing studies demonstrated that the Dalmatian dog did not differ from the non-Dalmatian in the production or output of total purine end-products, for the concentration of total purine end-product

nitrogen in the blood and the total quantity excreted in the urine were almost identical.

Moreover, there was no *qualitative* difference in the purine metabolism of the two varieties of animals, but a distinct, although comparatively minor, quantitative difference was found. That is, the Dalmatian dog, although able to form allantoin, was observed to be unable to convert uric acid into this substance as readily as the non-Dalmatian. This relative defect in the oxidation of uric acid was small enough in one of our dogs to make it easily understandable why former efforts to detect changes in the uricase content of the Dalmatian liver might have been inconclusive.

The inability of the Dalmatian to oxidize uric acid into allantoin as readily as the non-Dalmatian dog might explain the higher uric acid in the plasma of the former animal. It would not explain, however, the tremendous amount of uric acid found in the Dalmatian's urine, for, as has been pointed out, the amount excreted was much more than was found in the non-Dalmatian dog whose plasma uric acid had been elevated artificially to a level above that of the Dalmatian's. Moreover the Dalmatian excretes more uric acid per kilo of body weight than does man (1), although the latter's blood contains about 10 times more uric acid per cc. than the Dalmatian's.

The chief reason for the presence of excess uric acid in the urine of the Dalmatian was discovered in this study to be due to the peculiar fact that it has the ability, unlike any other known animal, to excrete uric acid at the exact level of glomerular filtration. This finding of ours is inferentially confirmed by the observations of both Young *et al.* (10) and Myers and Hanzal (11), for, in their published data, the daily creatinine and uric acid excretions of the Dalmatian dog were approximately the same. Since the creatinine and uric acid concentrations in the Dalmatian's blood are approximately equal, their equal excretion of both substances suggests the similarity or equality of their respective clearances.

This inability on the part of the Dalmatian dog to reabsorb uric acid from the glomerular filtrate undoubtedly results in a condition in which less uric acid is available in its body for conversion to allantoin and hence less allantoin is formed and excreted. The experimental data bear out this last assumption in that the allantoin excretion of the Dalmatian was decreased to the same extent that the uric acid excretion was increased, with the net result that the excretion of *both* purine end-products in the Dalmatian was the same as that of the non-Dalmatian. Furthermore when the renal escape of uric acid was prevented in the Dalmatian, it was found that the retained uric acid was converted almost completely into allantoin. These facts make it seem quite likely that the fundamental anomaly of the Dalmatian is its inability to reabsorb uric acid from the glomerular filtrate.

It is quite possible that, because of the unique ability of the Dalmatian kidney to excrete uric acid as efficiently as allantoin (*i.e.* at the level of glomerular filtration (8)), there is less biological necessity for the conversion of uric acid into allantoin. This rapid loss of uric acid by the Dalmatian kidney, then, may have led to a state in the process of this dog's evolutionary development which allowed a relative physiological deficiency in the oxidation of uric acid. Certainly it seems clear that the chief anomaly of the Dalmatian dog resides in the kidney, whereas the deficiency in its uricase system appears to be merely relative and possibly only a secondary effect of the initial renal oddity.

SUMMARY

1. The Dalmatian dog converted blood uric acid into blood allantoin less readily and completely than did the non-Dalmatian. This, however, was a minor quantitative difference when considered in regard to its effect on uric acid excretion.

2. The principal cause for the occurrence of large amounts of uric acid in the urine of the Dalmatian was found to be due to the fact that uric acid was excreted by this animal at the level of glomerular filtration without subsequent tubular reabsorption or excretion. This inability to reabsorb uric acid results in decreased retention of the latter substance in the body and hence decreased production and excretion of allantoin. The total production and excretion of *both* purine end-products, however, was the same in the Dalmatian and non-Dalmatian dog.

The authors wish to express their thanks to Catharine Shuey and Barbara Trousdale for technical assistance.

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THE URINARY EXCRETION OF CITRATE IN URANIUM-POISONED RATS*

By FRANCES L. HAVEN AND CHALLISS RANDALL

(From the Department of Biochemistry and Pharmacology, School of Medicine and Dentistry, The University of Rochester, Rochester, New York)

(Received for publication, May 13, 1948)

The increased urinary excretion of citrate following administration of sodium bicarbonate has been noted by many investigators (1-9). During the course of work on the effect of uranium on various metabolic processes we wished to ascertain whether the kidney poisoned by uranium would excrete citrate to the same extent that a normal kidney does after administration of sodium bicarbonate. Surprisingly, an increased excretion of urinary citrate was found to occur following a single sublethal dose of uranium nitrate. Studies of the urinary excretion of citrate following (a) a single dose, (b) increased doses, and (c) repeated small doses of uranium nitrate and the possible relationship of such excretion to the phenomenon of tolerance constitute the substance of this paper.

EXPERIMENTAL

Adult Wistar strain rats were used throughout this investigation. Each rat was housed in a wire mesh cage on top of a glass funnel containing wire screening for separation of feces. Beneath the funnel stem, a graduated cylinder containing toluene served for collection of urine. Urinary volume, pH, and citric acid content were determined on 24 hour samples.

Citric acid was determined by the method of Pucher, Sherman, and Vickery (10) modified as follows: (1) the oxidation mixture was decolorized with 3 per cent hydrogen peroxide at 10°; (2) the decolorized samples were shaken vigorously for 1 minute with petroleum ether for complete removal of pentabromoacetone from the aqueous layer (11); (3) petroleum ether was distilled once, allowed to stand several days over concentrated sulfuric acid, washed free from acid with distilled water, and redistilled; (4) dioxane was used as the color stabilizer (12); (5) color was developed by use of a sodium sulfide solution consisting of 4 gm. of $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ per 100 ml. of water.

* This paper is based on work performed under contract No. W-7401-eng-49 for the Atomic Energy Project at The University of Rochester and is taken from the thesis submitted by one of us (C. R.) to The University of Rochester in partial fulfillment of the requirements for the degree of Master of Science, June, 1946.

Presented at the Thirty-seventh annual meeting of the American Society for Pharmacology and Experimental Therapeutics at Chicago, May, 1947.

Among numerous substances tried by Pucher and others, the only one found to enhance the color given by citric acid in this method was β -hydroxybutyric acid. In view of this fact, determinations of β -hydroxybutyric acid were carried out by the method of Behre (13) on samples of rat urine known to be high and low, respectively, in citric acid. No β -hydroxybutyric acid was found; therefore, it may be assumed that this substance did not contribute to the values reported as citric acid.

All doses of uranium nitrate, $\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, were administered intraperitoneally in aqueous solution. The *single dose* consisted of 2.5 mg. of the hexahydrate per kilo, a sublethal dose. When *increased doses* were to be given, an initial dose of 0.5 mg. of the salt per kilo was administered. 14 days later a second dose of 0.5 mg. per kilo was given. Thereafter the dose was doubled every 12 days until the rat died. By this method toler-

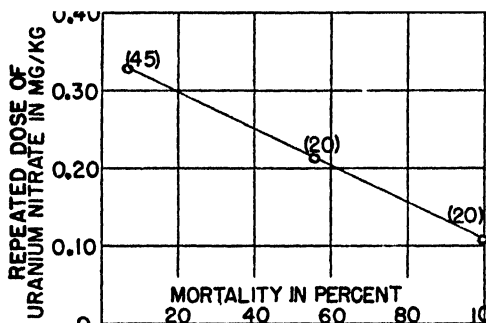


FIG. 1. Relationship between the mortality following an intraperitoneal test dose (5 mg. per kilo) of uranium nitrate and the size of the preceding repeated intraperitoneal dose in adult female rats. The number of animals per point is given in parentheses.

ance to ordinarily fatal doses has been produced in rats. *Repeated small doses* of two different sizes were employed; one consisted of 0.33 mg. per kilo, an amount found to protect rats from a *test dose* of 5 mg. per kilo, which would have been lethal had no previous treatment been given; the other consisted of 0.11 mg. per kilo, which had been found to be too small to prevent mortality following the test dose (Fig. 1). The repeated doses were given in a program of eleven injections, one every other day for 22 days.

Results

Excretion of Citrate Following Single Dose of Uranium Nitrate—The excretion of citrate (mg. of citric acid per 24 hours) and the body weight for a typical rat before and after 2.5 mg. per kilo of uranium nitrate was ad-

ministered are shown in Fig. 2. In 3 days after injection the excretion rose to 3 to 4 times the normal level. By the 6th day excretion had returned to normal. This was followed by a secondary rise 4 days later to 3 to 4 times the normal level, and was maintained for at least 8 days. The body weight decreased from the time the animal received the dose of uranium nitrate until the secondary rise in citrate excretion began and then increased.

Excretion of Citrate during Administration of Increased Doses of Uranium Nitrate—The excretion of citrate for one rat before and during the admin-

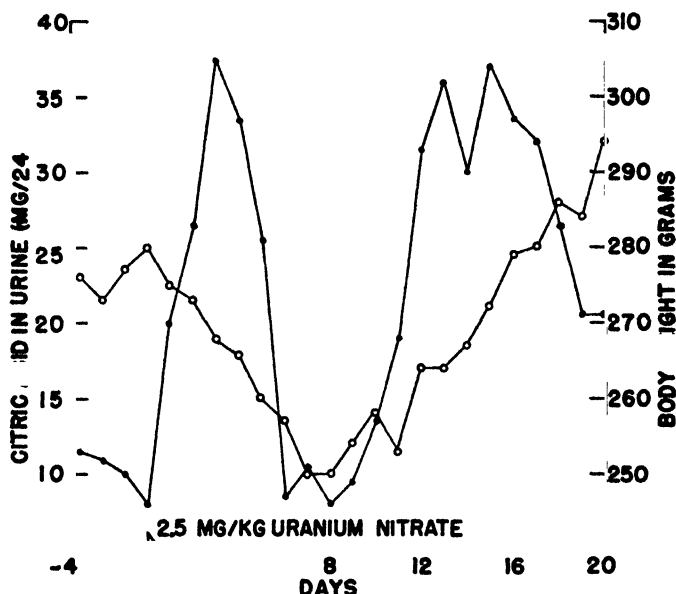


FIG. 2. The urinary excretion of citrate expressed as citric acid (mg. per 24 hours) (●) and the body weight (gm.) (O) of an adult male rat following the intraperitoneal administration of 2.5 mg. per kilo of uranium nitrate.

istration of increased doses of uranium nitrate is shown in Fig. 3. The output of citrate increased following the initial dose of 0.5 mg. per kilo and was significantly higher than normal each time a larger dose was given. The final dose of 16.0 mg. per kilo was 3 times the fatal single dose of 5 mg. per kilo; thus a high degree of tolerance had been attained. Similar results were obtained for a second rat.

Excretion of Citric Acid during Administration of Repeated Small Doses of Uranium Nitrate—The excretion of citrate during repeated administration of 0.33 mg. per kilo to one rat and of 0.11 mg. per kilo to another rat is shown in Fig. 4. During repeated injections of 0.33 mg. per kilo the ex-

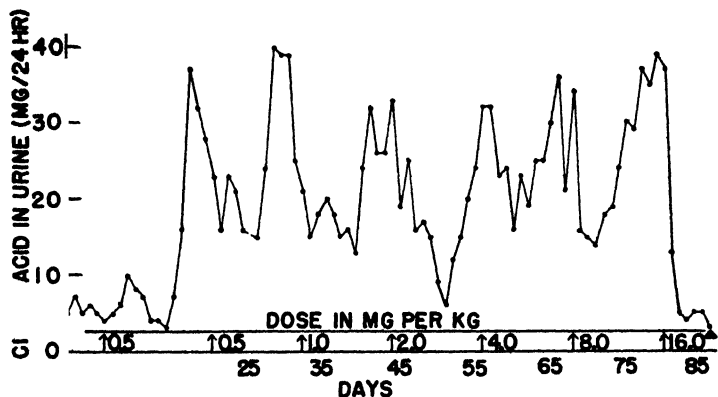


FIG. 3. The urinary excretion of citrate expressed as citric acid (mg. per 24 hours) of an adult male rat during the intraperitoneal administration of increased doses of uranium nitrate. Δ = death.

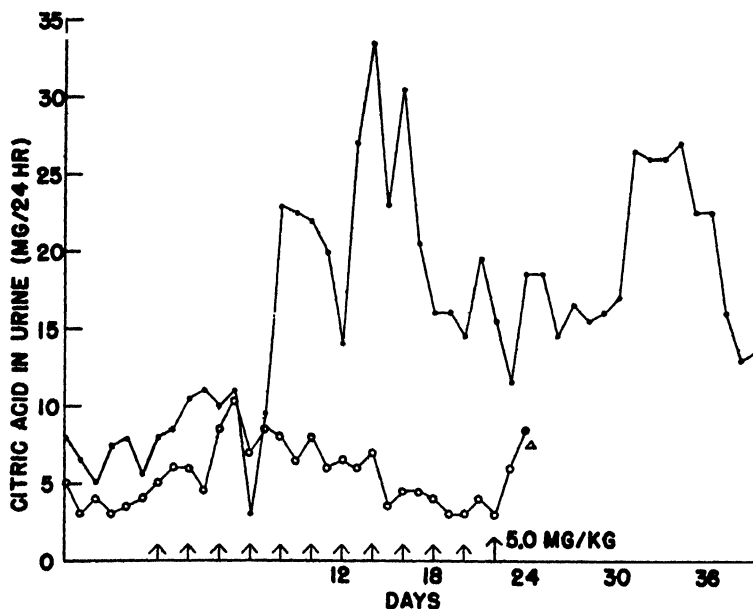


FIG. 4. The urinary excretion of citrate expressed as citric acid during repeated dosage with uranium nitrate. ● = 0.33 mg. per kilo, ○ = 0.11 mg. per kilo, Δ = death.

cretion rose and was about twice the normal at the time the test dose of 5 mg. per kilo was given. On the other hand, the rat that received repeated doses of 0.11 mg. per kilo exhibited only a transitory rise in excretion of

citrate, which had returned to the normal level by the time the test dose of 5.0 mg. per kilo was given. Tolerance was shown by the first rat, since it survived the test dose, while the second rat developed no tolerance and died. Similar results were obtained on two other rats given small doses of 0.33 and 0.11 mg. per kilo, respectively.

DISCUSSION

According to current views (14) of the mechanism of uranium poisoning, a part of the uranium is carried in the blood as the diffusible bicarbonate complex which passes through the glomerulus of the kidney and into the tubule. In progressing toward the distal end of the proximal third of the tubule the urine becomes more acid, owing to the resorption of base and bicarbonate without an accompanying resorption of non-volatile acid. Since the uranium-bicarbonate complex is unstable below pH 6.5, as the pH of the urine drops below this value uranium is freed to combine with protein of the tubule surface and cause damage.

The administration of carbonate to dogs (15) and of bicarbonate to rats¹ (16) has been shown to prevent the toxicity of uranium nitrate. Likewise, sodium citrate administered either intravenously or orally to uranium-poisoned dogs promoted survival and ameliorated the symptoms of poisoning (17-19). Oral administration of sodium citrate to rats was found by us to decrease the mortality to a dose of 5 mg. of uranium nitrate per kilo.

Administration of sodium bicarbonate or of sodium citrate would tend to keep the urinary pH high enough so that uranium would be present as the bicarbonate complex. Alkali administration would also increase urinary bicarbonate and citrate ions, which could form a complex with uranyl ions to prevent their combination with protein and consequent damage to the kidney tubule.

The strong ability of citrate to form complexes with the uranyl ion has been repeatedly demonstrated (20, 21). If the urinary pH should fall below 6.5, as often happens in uranium poisoning, the uranium set free from its complex with bicarbonate could be prevented from combining with renal protein by the presence in the kidney tubule of large amounts of citrate. In the rat, the ability of the kidney to synthesize citric acid (22) and, in the rabbit, to regulate the level of this substance in the blood serum (23) has been demonstrated.

Evidence in support of the assumption that the uranium-citrate complex is stable in the presence of protein was obtained from an experiment designed to imitate the flow of urine through the kidney tubule. Two solutions were prepared, each of which contained 200 mg. of dialyzed albumin,

¹ Haven, F. L., and Randall, C., unpublished work.

0.87 gm. of sodium chloride, and 600 γ of uranium. To the experimental solution were added 1980 mg. of citric acid. The pH was adjusted to 5.0 and the volume to 100 ml. Both solutions were filtered through a collodion membrane, and citric acid and uranium were determined on the protein-free filtrates. No uranium was found in the control filtrate, while in the experimental filtrate 81 per cent came through the membrane. Thus the uranium-citrate complex is diffusible, and citrate can prevent the combination of uranium with protein at a pH as low as 5.0. Rat urines after poisoning have never been more acid than this.

In the light of the evidence presented above citric acid might play an important rôle in tolerance to uranium. If citrate in the kidney tubule is effective in combating uranium poisoning, rats injected with uranium nitrate when the urinary citrate is elevated should be able to survive larger doses of the poison. Both indirect and direct evidence in support of this assumption has been obtained.

In experiments designed to test the best method of making rats tolerant to fatal doses of uranium nitrate, increased doses were given to one group at 10 day intervals and to a second group at 20 day intervals. The 10 day interval proved to be superior to the 20 day interval for producing tolerance. Examination of Fig. 2 reveals that at 10 days after a single injection of uranium nitrate the excretion of citrate had begun to rise, while at 20 days the excretion was falling. More direct evidence is obtained from Fig. 3, where it will be seen that each time an increased dose was given the excretion of citrate was high.

In spite of the fact that the excretion of citrate was high when each dose was given (Fig. 3), no greater percentage of the dose was excreted in the 48 hour period after each increased dose than in the same period after a single dose. Moreover, the kidneys of rats made tolerant by repeated doses have been found to contain significantly smaller amounts of uranium, expressed as percentage of the total dose, than the kidneys of rats given a single dose. Since the amount of uranium excreted in the urine is dependent on the amount which filters through the glomerulus, the presence of citrate in the tubule would not be expected to increase the excretion of uranium but would prevent the toxic action of uranium by preventing its combination with protein. Because uranium is not excreted in larger proportions and is present in kidney in lesser amounts in "tolerant" rats than following a single dose, it is logical to assume that the uranium-citrate complex formed in the tubules is returned for the most part to the blood where the uranium, having formed a complex either as bicarbonate or citrate, would be carried to the bones and deposited (14).

Additional evidence in support of the postulated rôle of citrate in tolerance is obtained from the studies of citrate excretion following repeated

doses (Fig. 4). During the repeated dosage with 0.33 mg. of uranium nitrate per kilo, a dose and method known to produce tolerance to uranium, as is shown in Fig. 1, the excretion of citrate rose and was about twice the normal when the test dose of 5 mg. per kilo was given. On the other hand, the dose of 0.11 mg. per kilo which was too small to produce tolerance (Fig. 1) caused only a transitory rise in excretion of citrate which had returned to normal by the time the fatal dose of 5 mg. per kilo was given.

In conclusion, the available evidence indicates that the presence of citrate in the kidney tubule in concentrations above normal may account for acquired tolerance to the toxic action of uranium.

SUMMARY

1. Following administration to the rat of a single intraperitoneal dose of 2.5 mg. of uranium nitrate per kilo, the urinary excretion of citrate rose in 3 days to 3 to 4 times the normal level; return to the normal level was followed by a secondary rise of equal magnitude.

2. When a rat received increased doses of uranium nitrate at 12 day intervals, the urinary excretion of citrate was high each time a larger dose was given.

3. At the time of administration of 5 mg. of uranium nitrate to a rat that had previously received eleven injections of 0.33 mg. per kilo (a dose which causes tolerance) the excretion of citrate was twice the normal; when the repeated small dose consisted of 0.11 mg. per kilo (a dose too small to cause tolerance), the urinary excretion of citrate was normal at the time the dose of 5 mg. per kilo was given.

4. As an explanation for acquired tolerance to uranium, high concentrations of citrate within the kidney tubule appear to result in formation of a soluble complex with the metal and thus protect against the toxic action.

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MICROESTIMATION OF CITRIC ACID; A NEW COLORIMETRIC REACTION FOR PENTABROMOACETONE*

By SAMUEL NATELSON, JOSEPH B. PINCUS, AND JULIUS K. LUGOVOY

(From the Department of Biochemistry and the Pediatric Research Laboratory, The Jewish Hospital of Brooklyn, New York)

(Received for publication, April 5, 1948)

In an earlier publication¹ a method was described for the determination of citric acid in blood serum for amounts as low as 5 γ . While this method permitted determinations on from 0.5 to 1.0 ml. of serum, a need was felt for a method which would be suitable for smaller amounts for children. This need was felt most acutely when samples were drawn at frequent intervals from the same child.

The extinction coefficient for the sodium sulfide color with pentabromoacetone is relatively low, $E_{1\text{ cm.}}^{1\%} = 92$. The development of a new colorimetric reaction with a higher extinction coefficient was of critical importance in our studies.

Investigation of various substances available containing sulfur indicated that thiourea and substituted thiourea compounds form colored complexes with pentabromoacetone. For example, if a petroleum ether solution of pentabromoacetone is added to an alcoholic KOH solution containing thiourea, acetylthiourea, ethylthiourea, allylthiourea, *sym*-diethylthiourea, or *sym*-dimethylthiourea, yellow to greenish yellow colors are observed in the alcohol layer. In this strongly alkaline solution the colors slowly fade at room temperature.

Thiourea was chosen for study because of its solubility in water, ease of purification, and availability.

A 4 per cent thiourea solution dissolved in water exhibits a pH of 6.9. When this solution is shaken with a petroleum ether solution of pentabromoacetone for 15 to 30 minutes, a pinkish color, resembling a dilute permanganate solution, develops in the aqueous phase. This color has maximum absorption at 510 $m\mu$. Addition of acid such as dilute acetic acid destroys the color. If the pink solution is now made alkaline to pH 11, a yellow color develops which reaches its maximum intensity after 7 to 15 minutes, with a maximum absorption at 460 $m\mu$. Both colors are stable for at least an hour at room temperature. If the pentabromoacetone solution is too concentrated, a precipitate of the pink complex will form.

* Presented at the meeting of the American Chemical Society at New York, September 15, 1947.

¹ Natelson, S., Lugovoy, J. K., and Pincus, J. B., *J. Biol. Chem.*, 170, 597 (1947).

Although the pink-colored complex is converted to the yellow compound on addition of alkali, these compounds are not in dynamic equilibrium with each other. Addition of alkali causes the disappearance of the 510 m μ peak, but the 460 m μ peak does not form until several minutes later.

For the purpose of analytical determination it is apparent that the pH had to be controlled in order to obtain a color with consistent extinction coefficient at one of the peaks. A procedure was developed, therefore, for the determination of citric acid in serum with the 460 m μ peak and pH 11.

In one procedure developed, citric acid is converted to pentabromoacetone as previously described,¹ and the pentabromoacetone is extracted from the petroleum ether layer with a 4 per cent thiourea solution in a buffer of pH 7. Before reading the color, the pH is brought to 11 with NaOH solution. For micro quantities (1 to 10 γ) of citric acid the colors are read in the Coleman spectrophotometer with the 3 ml. capacity cuvettes and a 5 cm. light absorption path. For the Beckman spectrophotometer the color is developed in 1 ml. of solution and is read in the micro cups.

A modification which has recently shown itself to be suitable and simpler is to extract the pentabromoacetone into a solution containing 4 per cent thiourea and 2 per cent borax or sodium pyrophosphate, the borax or sodium pyrophosphate maintaining the pH at 9.2. In this manner the extraction time is less and the color may be read directly without changing the pH. The peak with a borax buffer is at 445 m μ and not at 460 m μ as with the phosphate buffer at pH 11. The extinction coefficient, however, is approximately the same.

The extinction coefficient plotted against wave-length for the thiourea-pentabromoacetone colors is illustrated in Fig. 1 for pH 11, 9.2, and 7.

For micro quantities of citric acid (0.2 ml. of serum) commercially available 2 ml. Pyrex volumetric flasks, test-tube shape, with ground glass stoppers, were utilized for the extractions. A mark was scratched on these tubes at 0.4 ml. with a diamond pencil. The stoppers were ground in by hand, a Palo-Myers grinding paste being used to prevent leakage. Silicone grease was also used for this same purpose.

✓ A standard curve indicating that Beer's law is followed is shown in Fig. 2.

To prepare the standard curve, dilutions of the citric acid stock solution are made. The same procedure is followed as is described for blood, except that the precipitation with trichloroacetic acid and the boiling down are omitted. In determining the total amount of citric acid in the sample of unknown the results obtained from Fig. 2 must be multiplied by 6/5 to correct for the 5/6 aliquot taken after protein precipitation.

The curve as plotted from determinations on known amounts of citric acid actually represents 10/13 of the total amount of citric acid present, because a 10/13 aliquot of the petroleum ether, which contains the pentabromoacetone, is taken.

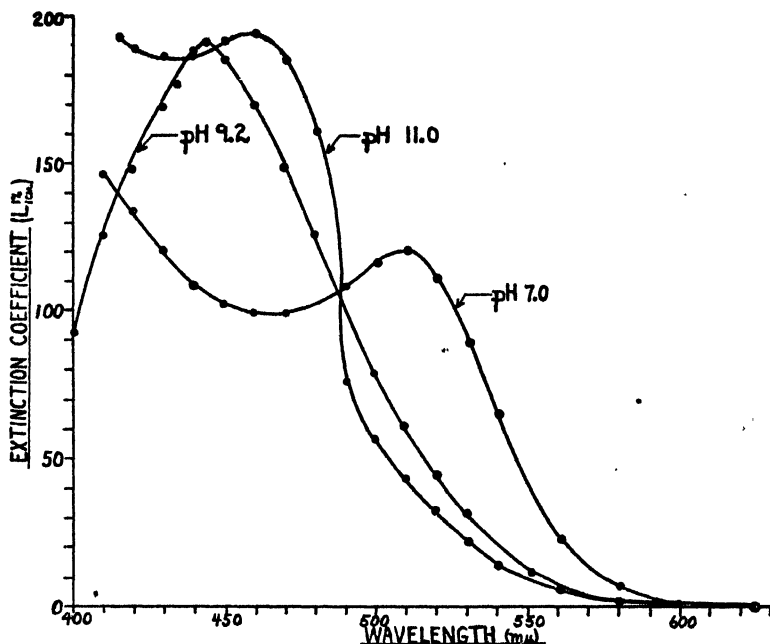


FIG. 1. Absorption spectra of the pentabromoacetone-thiourea complex at pH 7.0, 9.2, and 11.0 with 10 γ of pentabromoacetone made up in 3.5 ml. of the buffered 4 per cent thiourea solution.

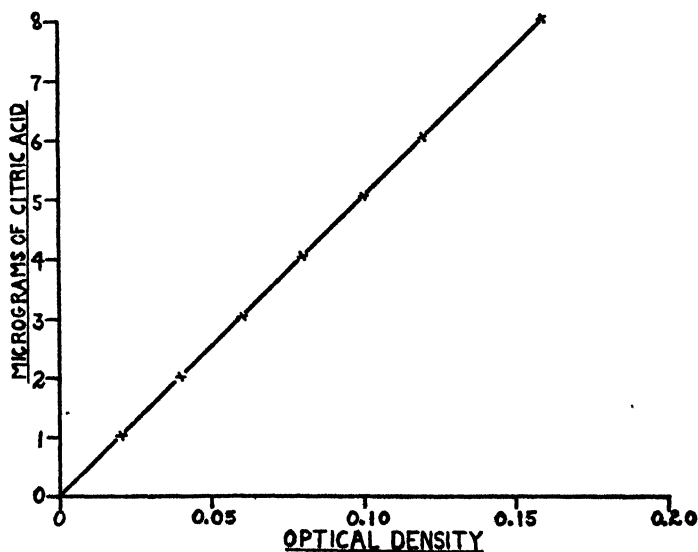


FIG. 2. Standard curve obtained by plotting optical density against concentration of citric acid at 445 $m\mu$ in the Coleman spectrophotometer with 3 ml. cuvettes with a 5 cm. light path; pH 9.2.

The procedures described have been used routinely in this laboratory for determination of citric acid for several months. When larger amounts of blood are available, the method may be adapted for determination in 1 ml. of serum. In this case the ordinary colorimeter (Klett) may be used with a No. 44 or No. 47 filter.

The specificity of the method is similar to that found for the sodium sulfide color. No measurable interference was found when aconitic acid, glucose, acetoacetic acid, hydroxybutyric acid, acetone, or pyruvic acid was added in amounts up to 50 mg. in determinations on 0.2 ml. of serum.

This procedure has advantages over the sodium sulfide method in that the color is stable at room temperature and smaller quantities may be determined, for the extinction coefficient is approximately twice that of the sodium sulfide color.

Typical results as obtained on four consecutive determinations are given in Table I.

TABLE I
Determination of Citric Acid in 0.2 Ml. of Blood Serum

Sample No.	Citric acid	Citric acid added	Citric acid recovered	Per cent recovered
	γ	γ	γ	
1	5.08	4.00	3.97	99.3
2	2.67	1.67	1.74	104.0
3	4.13	2.86	2.88	100.9
4	3.25	1.50	1.44	96.0

Method

Reagents—

Citric acid. Stock solution, 1 ml. = 1 mg. of anhydrous citric acid (analytical reagent), made up in 1 N H_2SO_4 . This solution is diluted 1:100 with water daily to prepare the dilute standard; 1 ml. = 10 γ .

Sulfuric acid. 18 N (analytical reagent).

Potassium bromide-bromine reagent. Distilled water is saturated with bromine. The saturated bromine water is decanted from the bromine. In this solution, KBr (reagent grade) is dissolved to make it 1 N with respect to KBr.

Hydrogen peroxide. 6 per cent, made by diluting 10 ml. of 30 per cent H_2O_2 (analytical reagent) to 50 ml. This solution is stored in a refrigerator.

Petroleum ether, b.p. 90–100°. The commercial product is allowed to stand over 0.1 of its volume of concentrated H_2SO_4 for several days. The

sulfuric acid is separated and the petroleum ether is washed several times with fresh sulfuric acid until the washings are colorless. The petroleum ether is shaken with saturated KMnO_4 solution made up in 0.5 N H_2SO_4 . The mixture is shaken and allowed to stand for 30 minutes and the KMnO_4 is washed out with water. The petroleum ether is allowed to dry over K_2CO_3 (anhydrous), filtered, and distilled, the 90–100° fraction being collected.

KMnO₄ solution. 5 per cent (reagent grade).

Thiourea solution, pH 9.2. 2 gm. of Borax (analytical grade) are dissolved in 100 ml. of 4 per cent thiourea.

Procedure—0.2 ml. of serum is placed in a 3 ml. centrifuge tube and 1 ml. of 10 per cent trichloroacetic acid is rapidly blown in to precipitate the proteins in fine particles. Towards the end, the pipette is allowed to drain in order to obtain an accurate measurement. The mixture is shaken and allowed to stand for 10 minutes. The tubes are stoppered and then centrifuged at 2500 R.P.M. The supernatant liquid is poured off as completely as possible into a clean test-tube, without disturbing the precipitate. A 1 ml. aliquot is taken and placed in a 3 ml. test-tube with a ground glass stopper (2 ml. Pyrex volumetric flasks, test-tube shape) with a mark at 0.4 ml. 0.04 ml. of 18 N H_2SO_4 is added and the solution is evaporated to the 0.4 ml. mark by placing the tube in an oil bath maintained at 100–120°. 0.04 ml. of the KBr-bromine solution is added to the cooled solution and the mixture is allowed to stand for 10 minutes. 0.1 ml. of the 5 per cent KMnO_4 solution is added. The tube is shaken and allowed to stand for 10 minutes. It is then cooled to approximately 10° by placing the tubes in the ice box or in an ice bath. The excess permanganate is decolorized with approximately 2 drops (0.03 to 0.06 ml.) of cold 6 per cent hydrogen peroxide. 1.3 ml. of the purified petroleum ether is added to the tube from a burette, and the tube is stoppered with a minimum of silicone grease. The pentabromoacetone is then extracted by shaking in a machine for 10 minutes. The tube is centrifuged for 5 minutes at 2000 R.P.M. A 1.0 ml. aliquot of the petroleum ether layer is taken and placed in a 7 to 12 ml. glass-stoppered centrifuge tube.

3.5 ml. of the thiourea solution, pH 9.2, are added, and the tube is stoppered and shaken in a shaking machine for 5 minutes. The tube is then centrifuged at 2000 R.P.M. for 5 minutes. The petroleum ether layer is aspirated off and enough of the aqueous phase to be read is pipetted into the 3.0 ml. cuvettes with a 5 cm. light absorption path. The density is read in the Coleman spectrophotometer at 445 μ . The color is read against a thiourea buffer solution which has been treated in a manner similar to the unknown (*i.e.*, extracted with petroleum ether and centrifuged). A reading is taken at 650 μ so as to correct for the difference in cloudiness be-

tween the unknown and blank. Most often the cloudiness correction is negligible.

Two standards, 4 and 6 γ content, are run to check the standard curve with each set of determinations. The slope of the standard curve may vary slightly from day to day if marked changes in room temperature occur or if the pH of the buffer should change. This is noted and corrected for by the use of the standards.

SUMMARY

1. A colorimetric method is described for determining citric acid in biological fluids. Citric acid is converted to pentabromoacetone which is allowed to react with thiourea.

2. Thiourea and substituted thiourea compounds produce colored complexes when allowed to react with pentabromoacetone. The nature of the color produced varies with the pH of the solution.

3. The method is accurate within 5 per cent for amounts ranging from 1 to 20 γ of citric acid as determined by recoveries added to serum. The intensity of the colors obtained with change in concentration follows Beer's law.

BIOSYNTHESIS OF PENICILLINS

I. BIOLOGICAL PRECURSORS FOR BENZYL PENICILLIN (PENICILLIN G)

By OTTO K. BEHRENS, JOSEPH CORSE,* REUBEN G. JONES,
MARJORIE J. MANN, QUENTIN F. SOPER, F. R. VAN
ABEELE, AND MING-CHIEN CHIANG†

(From the Lilly Research Laboratories, Indianapolis)

(Received for publication, February 28, 1948)

Early work on the structure of penicillin quickly yielded information concerning the identity of the fragments which may be obtained through hydrolytic cleavage of penicillin. Such knowledge made possible a systematic study of the course of penicillin biosynthesis. It seemed probable that the fermentative production of penicillin might be limited by the capacity of the mold to form adequate amounts of essential intermediates. To test this thesis a comprehensive study was begun to determine whether degradation products, proposed metabolic intermediates, or similar substances might be capable of stimulating the production of penicillin by the mold by acting as precursors. In this search we have been successful; the penicillin yield may be substantially increased by making certain additions to the media.

Studies carried out in the Northern Regional Research Laboratory (1) had previously shown that the addition of small amounts of phenylacetic acid to the medium stimulated the production of penicillin in surface cultures but had little effect on the yields obtained in submerged cultures. An effort by these workers to demonstrate an influence on the type of penicillin produced was unsuccessful (1).¹

This effect of phenylacetic acid was regarded in many quarters as a probable stimulation by a plant hormone-like substance. In contrast, we held it likely that phenylacetic acid acted as a precursor in surface culture, and it was suggested that some other substances would fulfill a similar function for submerged cultures. A considerable number of derivatives of phenylacetic acid have been found to be effective in stimulating the production of penicillin in submerged cultures. As has been pointed out, phenylacetic

* Present address, University of California, Los Angeles, California.

† Present address, Department of Chemistry, National University of Peking, Peiping, China.

¹ It is evident that direct utilization of phenylacetic acid by the mold for penicillin formation will lead to the formation of benzylpenicillin, in which the acyl portion of the molecule is the phenylacetyl group.

acid was not effective in submerged cultures with mold strains tested at the inception of this work. However, following the introduction of new and higher yielding strains, many compounds were retested, and it was found that phenylacetic acid is utilized by some of these strains (e.g. *Penicillium chrysogenum* Q-176, X1612).

Early efforts to obtain leads concerning the types of compounds that could influence penicillin production were made with strain NRRL 1976. For these tests a suboptimal synthetic medium was used, designed to supply sources of energy for the mold but not to allow optimal penicillin formation. Control flasks usually produced about 15 to 20 units of penicillin per ml.² in 48 hours. The test compound was added to such a medium in 0.0008 M concentration. Complete conversion of the precursor to penicillin would yield a broth assaying 475 units per ml.; utilization of only one form of a racemic compound would yield one-half this value. A washed suspension of the mold, which had been grown in corn steep medium, served as the inoculum. This test method was designated the "low" (L) method.

It was realized, however, that a stimulation of yield on such a medium could be interpreted as being due to improvement of a suboptimal medium rather than to the effect of a direct precursor. For this reason a second test, designated the "high" (H) method was used. The medium was more complete. It included corn steep solids, and normally yielded 100 to 120 units per ml. In general, the two methods gave comparable results; i.e., compounds that stimulated production in Method L also stimulated production when tested by Method H. Additionally, it was proposed to use substances containing isotopic elements to obtain direct proof that the stimulating compounds actually functioned as penicillin precursors. These latter experiments are described in Paper II of this series.

Table I presents the effect of a number of compounds on the yield of penicillin. More extensive data may be found in the monograph on penicillin (2).

Marked specificity was evident in the ability of the mold to utilize compounds. This specificity applied to both the acyl and amide portions of the test compounds. It has been determined that differences in the acyl portion of the molecule are of importance because of limitations in the mold's metabolic ability to incorporate these groups into the penicillin molecule. We may therefore conclude that under the conditions used in these tests the mold probably does not form penicillins which contain benzoyl or β -phenylpropionyl groups. However, in Table I it is seen that a γ -phenylbutyryl compound provided good stimulation in yield. The peni-

² Assays were obtained by the plate method with *Staphylococcus aureus*, strain 209P. We acknowledge with thanks the numerous assays performed by Dr. J. M. McGuire. The method is described in detail in the penicillin monograph (2).

cillin formed when N-(2-hydroxyethyl)- γ -phenylbutyramide was used as the precursor was isolated and identified as benzylpenicillin. This was interpreted as evidence that phenylbutyryl compounds are degraded by the mold with the loss of 2 carbon atoms. Proof of this interpretation is provided in Paper IV in this series (3).

TABLE I

Effect of Various Compounds on Penicillin Production

The tests were performed with strain NRRL 1976. Compounds were added at 0.0008 M concentration. The numerical values represent the ratio, units in test container to units in control container. The following values were obtained with the "high" method.

N-Phenylacetyl-L-valine	1.42
Phenylacetic acid + DL-valine	1.0
N-Phenylacetyl-D-valine	1.0
N-Phenylacetyl-L-alanine	1.0
N-Phenylacetyl-glycine + N-acetyl-DL-valine	1.0
N-Benzoyl-DL-valine	1.0
N- β -Phenylpropionyl-DL-valine	1.0
N- γ -Phenylbutyryl-DL-valine	1.26
N-(2-Hydroxyethyl)-phenylacetamide	1.57
N-(2-Aminoethyl)-phenylacetamide	1.56
2-Aminoethyl phenylacetate hydrochloride	1.26
N-Allylphenylacetamide	1.48
N-Crotylphenylacetamide	1.94
Phenylacetylated pancreatic digest of casein (125 mg. %).	1.46
N- β -Methylallylphenylacetamide	1.24
N- γ , γ -Dimethylallylphenylacetamide	1.21
N-(2-Acetoxyethyl)-phenylacetamide	1.41
N-(2-Butyroxethyl)-phenylacetamide	1.46
N-(2-Isocaproxyethyl)-phenylacetamide	1.29
N-(2-Ethoxyethyl)-phenylacetamide	1.20
N-(2-Pentenyl)-phenylacetamide	1.28
N-(2-Ethyl-2-hydroxybutyl)-phenylacetamide	1.0
N-(2-Hydroxy-2-phenylethyl)-phenylacetamide	1.0
Butyl DL- α -phenylacetyl-amino- <i>n</i> -valerate	1.15
N-(2-Hydroxy-2-methylpropyl)-phenylacetamide	1.1

The reasons for the marked specificity of the nitrogen moiety have not been determined. It has become evident that in these compounds this portion of the molecule is not directly incorporated into the penicillin. Its function, therefore, appears to be an indirect one and may be concerned with the availability of the acyl group to the mold.

The effect of variation in the concentration of a precursor is illustrated in Table II. At concentrations of 0.01 per cent or less, it was still possible

to demonstrate, by the differential assay method,³ that the precursor affected the type of penicillin produced.

Many of the compounds which were tested as precursors for benzylpenicillin do not appear to have been previously described. They are presented in Table IV.

EXPERIMENTAL

Methods of Testing Precursors

Low (L) Method—The vegetative inoculum was grown in cotton-stoppered 1 liter Erlenmeyer flasks containing 200 ml. of a culture medium consisting of 20 gm. of lactose, 20 gm. of corn steep solids (American Maize Products), 0.5 gm. of monopotassium phosphate, 0.25 gm. of magnesium sulfate heptahydrate, 2 gm. of sodium nitrate, and 0.02 gm. of zinc sulfate heptahydrate per liter. Seeding was accomplished with 0.5 ml. of a spore suspension of *Penicillium notatum*, strain NRRL 1976. As a source of

TABLE II
Effect of N-Phenylacetyl-DL-valine on Strain NRRL 1976

Concentration	Molarity	Units per ml.
<i>per cent</i>		
Control	0	119
0.01	0.00038	129
0.02	0.00076	195
0.04	0.00152	182

spores the mold was grown on Moyer and Coghill's sporulation medium (4) in test-tube slant cultures. Suspensions of the spores were prepared by adding 10 ml. of water and brushing off the spores with a platinum wire. The culture was incubated for 3 to 5 days at 24° with continuous agitation on a reciprocating shaker (3 inch stroke, 100 strokes per minute). At the end of this period the broth was removed from the well formed pellets by sterile filtration. The pellets were washed once with sterile water and were suspended in 100 ml. of a medium containing 1 gm. of monopotassium phosphate, 1 gm. of dipotassium phosphate, 1 gm. of magnesium sulfate heptahydrate, 2 gm. of sodium nitrate, 10 gm. of lactose, and 0.01 gm. of zinc sulfate heptahydrate per liter and adjusted to pH 6.5.

The pellets and medium from one flask were subdivided into four equal portions and were placed in 300 ml. Erlenmeyer flasks. To one of these flasks, used as a control, were added 5 ml. of 0.02 M phosphate buffer, pH

³ The differential assay is the ratio of antibacterial activity for *Bacillus subtilis* to *Staphylococcus aureus* compared with that for pure benzylpenicillin, which is defined as 1.00.

6.5, and 15 ml. of water. To the other three flasks were added the three sets of constituents to be tested. Each of the substances (0.00004 mole) to be tested in a given flask was dissolved in 5 ml. of 0.02 M phosphate buffer, pH 7.0, and 5 ml. of water, and was adjusted to pH 6.5. This test solution was introduced into the Erlenmeyer flask through a Seitz filter, followed by 10 ml. of water. The set of flasks was shaken at 24° and samples were removed at suitable intervals for testing. Duplicate experiments were performed.

High (H) Method—100 ml. of medium (containing 25 gm. of lactose, 20 gm. of American Maize corn steep solids, 2 gm. of calcium carbonate, and 0.044 gm. of zinc sulfate heptahydrate per liter) in a 1 liter Erlenmeyer flask were inoculated with 1.0 ml. of a spore suspension of strain NRRL 1976. The flask was shaken for 2 days at 24°. 10 ml. of the germinated spores were then introduced into each of a series of 300 ml. Erlenmeyer flasks containing 40 ml. of a medium, double the concentration of constituents in the original flask, to which had been added 25 ml. of water and 5 ml. of 0.02 M phosphate buffer, pH 6.5, containing 6.4×10^{-5} mole of the test substance. Control flasks without addition of precursors generally yielded 100 to 120 units per ml. in 4 or 5 days.

Penicillin from N-(2-Hydroxyethyl)- γ -phenylbutyramide—The precursor (166 mg.) was added to a broth containing 25 gm. of lactose, 20 gm. of corn steep solids, 2 gm. of calcium carbonate, and 0.044 gm. of zinc sulfate heptahydrate per liter. 225 ml. of broth in each 1 liter Erlenmeyer flask were inoculated with 0.5 ml. of a spore suspension of *Penicillium notatum*, strain NRRL 1976. The flasks were shaken at 25° for 5 days and harvested. 5.4 liters of cold filtered broth, 150 units per ml., were extracted at pH 2.2 with 3.2 liters of cold amyl acetate. The amyl acetate was separated (in some instances the emulsion separated on standing for $\frac{1}{2}$ hour; in other cases use was made of a small Sharples supercentrifuge), and the sodium salt was prepared by stirring with three 50 ml. portions of 0.1 N sodium bicarbonate solution. The pH values of the aqueous extracts were in the range 6.9 to 7.2. An ethereal solution of the penicillin was prepared by acidifying the chilled aqueous solution to pH 2.1 with 10 per cent phosphoric acid and extracting with three 50 ml. portions of alcohol-free ether. Assay of a sample of the ethereal solution indicated the presence of 455,000 units of penicillin.

A column was prepared for chromatographic purification of the penicillin. 50 gm. of dry silica (a suitable material may be obtained from the G. Frederick Smith Chemical Company, Columbus, Ohio) were thoroughly mixed with 42.5 ml. of 1.5 M potassium phosphate buffer, pH 6.2. The silica, which still retained a dry appearance, was suspended in ether and transferred to a glass tube 1 inch in diameter. Glass wool was used at the bottom of the tube to retain the silica. Additional ether was used in transferring the silica and a few pounds of air pressure were applied to obtain proper

packing. Care was exercised to keep excess ether on the column at all times. The ethereal solution of the penicillin was carefully poured into the column, followed by successive 100 ml. portions of ether containing 0.5, 1, 1.5, 2, 2.5, and 3 per cent of methanol. The ether effluent was collected in 100 ml. portions. At the conclusion of the development of the column the ether was allowed to drain from the silica. The silica was removed in $\frac{1}{2}$ inch cuts and the penicillin was recovered from the portions of silica and ether by extraction with 0.067 M phosphate buffer of pH 7.0. Assays on the various fractions indicated the distribution shown in Table III.

Fractions S-6, S-7, and S-8, containing 60 per cent of the recovered units, were combined, extracted with three 40 ml. portions of cold chloroform at pH 2.2, and were further purified by use of a chloroform-phosphate buffer (pH 6.2) column. The development of the column was carried out with successive 100 ml. portions of chloroform containing 1, 2, and 3 per cent

TABLE III
Distribution of Penicillin from N-(2-Hydroxyethyl)- γ -phenylbutyramide on Ether-Buffer (pH 6.2) Chromatographic Column

Fraction	Units	Fraction	Units
S-1	14,600	S-10	40,000
S-2	5,000	S-11	28,000
S-3	5,000	F-7	40,400
S-4	None	F-6	7,500
S-5	5,400	F-5	6,600
S-6	92,000	F-4	5,000
S-7	152,000	F-3	None
S-8	42,600	F-2	"
S-9	47,000	F-1	"

methanol. 218,000 units were recovered in a single sharp band. The penicillin was extracted from the buffer solution with ether at pH 2.2 and the sodium salt was prepared with 0.1 N sodium hydroxide solution. The aqueous solution was dried from the frozen state, and the resulting dry sodium salt was treated with 1 ml. of acetone. Partial solution followed by reprecipitation occurred. This precipitate was washed with several portions of dry acetone and was crystallized from 2 ml. of 90 per cent acetone by addition of 4 ml. of dry acetone. It was recrystallized in a similar manner.

Analysis—Sodium γ -phenylpropylpenicillin, $C_{18}H_{21}N_2O_4SNa$

Calculated. C 56.23, H 5.51, N 7.31

Sodium benzylpenicillin, $C_{18}H_{17}N_2O_4SNa$

Calculated. C 53.92, H 4.80, N 7.83

Found. " 53.47, " 4.51, " 7.85

The material assayed 1630 units per mg. and gave a differential assay value of 0.99. These properties indicated that the product was benzylpenicillin.

Preparation of Phenylacetylated Protein Hydrolysates

Phenylacetylated Corn Steep Solids—20 gm. of American Maize corn steep solids were dissolved in 35 ml. of water. The solution was cooled well, made alkaline with 5 N sodium hydroxide solution, and 10 ml. of phenylacetyl chloride and 45 ml. of 2.5 N sodium hydroxide solution were added in portions with vigorous stirring over a period of $\frac{1}{2}$ hour. After an additional $\frac{1}{2}$ hour the mixture was acidified with 5 N hydrochloric acid and was shaken with several portions of ether. The ether extracts were combined, evaporated to dryness *in vacuo*, and the residue was dissolved in 50 ml. of ethyl acetate and precipitated with 50 ml. of petroleum ether. The oily precipitate was returned to the acid aqueous solution, which was then adjusted to pH 6.5 with 5 N sodium hydroxide solution.

Phenylacetylated Casein and Liver Hydrolysates—A pancreatic casein digest, a papain digest of casein, an acid-hydrolyzed casein, and a pancreatic digest of liver were each phenylacetylated in the manner described above for corn steep solids. 10 gm. of digest in 15 ml. of water were treated with 10 ml. of phenylacetyl chloride and alkali.

The above hydrolysates were prepared in the following manner.

Pancreatic Digest of Liver—4 kilos of freshly ground liver were incubated at 37° for 6 days with 800 gm. of freshly ground pancreas in a total volume of 16 liters. 53 gm. of sodium carbonate were added at the beginning of the reaction. Toluene and chloroform were added as preservatives. At the end of the incubation period, hydrochloric acid was added in an amount equivalent to that of the sodium carbonate, and the mixture was heated to 90–94° for 15 minutes. After the addition of 25 gm. of Nuchar, the mixture was filtered, and the filtrate was evaporated to dryness *in vacuo*.

Pancreatic Digest of Casein—150 gm. of casein were digested with 150 gm. of minced pancreas in a total volume of 3 liters. 19 gm. of sodium carbonate were added at the beginning of the reaction. The subsequent procedure was the same as that described for the liver digest above, except that no decolorizing carbon was used.

Acid-Hydrolyzed Casein—Casein (300 gm.) was hydrolyzed by refluxing for 21 hours with 5 volumes of 26 per cent sulfuric acid. The sulfuric acid was removed quantitatively by addition of barium hydroxide. The precipitated barium sulfate was removed by filtration and was thoroughly washed with hot water. The aqueous solution was decolorized with carbon and was evaporated to dryness *in vacuo*.

Papain Digest of Casein—United States patent 2,364,008 of E. H. Stuart.

Preparation of Miscellaneous Precursors—"Penillic acid" was prepared by treating 500 unit commercial penicillin with dilute hydrochloric acid

according to the directions for preparation of penillic acid from crystalline penicillin (2). The neutralized solution was used for the test.

"Penicilloic acid" was prepared from 500 unit commercial penicillin by treatment with dilute sodium hydroxide solution at pH 11.5 for 2 hours. The neutralized solution was used for the test.

Preparation of Benzylpenicillin Precursors—A number of different methods (as designated in Table IV) was employed in the preparation of the various compounds.

Method A—A methyl or ethyl ester of the acyl portion of the molecule was heated at 100–150° for several hours with a slight excess of the appropriate amine. The mixture was cooled and the product was recrystallized from appropriate solvents such as alcohol-ether, ethyl acetate, benzene-petroleum ether, etc. Those products which did not crystallize were purified as follows: The solution of the compound in a solvent such as ether or ethyl acetate was washed successively with dilute acid, dilute alkali, and water. The solution was dried, and the solvent removed, finally by heating in a high vacuum.

Method B—The regular Schotten-Baumann method was applied to the preparation of these compounds. N-Alkylphenylacetamides were purified as described under Method A. N-Phenylacetyl amino acids were purified by precipitation from acidified solution, followed by recrystallization from appropriate solvents.

Method C—A suspension of 22.5 gm. of N-allylphenylacetamide (Table IV) in 1800 ml. of water at 0° was treated dropwise with a solution of 10 gm. of potassium permanganate in 300 ml. of water. The mixture was filtered and the filtrate was evaporated to dryness *in vacuo*. The residue, 25.7 gm., was recrystallized from ethylene dichloride to yield 12.5 gm. of N-(2,3-dihydroxypropyl)-phenylacetamide containing 1 molecule of water of hydration.

Method D—A mixture of 29.6 gm. (0.4 mole) of trimethylenediamine, 34 gm. (0.25 mole) of phenylacetic acid, and 0.3 mole of 4 N hydrochloric acid was heated to 250° during 1 hour. The melt was cooled, dissolved in 300 ml. of water, and the solution was filtered and made alkaline with sodium hydroxide solution. The aqueous solution was extracted with ether and the ether extract was dried and evaporated, leaving N-(3-aminopropyl)-phenylacetamide as a crystalline solid.

Method E—To a solution of 27 gm. (0.2 mole) of phenylacetamide in 200 ml. of dioxane were added 7.8 gm. of potassium. After all of the metal had reacted, 18.1 gm. of β -methylallyl chloride were added, and the solution was heated under a reflux for 4 hours. The mixture was filtered and the filtrate was evaporated *in vacuo*. The residue was taken up in warm benzene. The solution was filtered, evaporated, and the residue recrystallized several

TABLE IV
Benzylpenicillin Precursors

Compound	Empirical formula	Method of preparation	M.p.	Nitrogen	
				Calculated	Found
			°C.	per cent	per cent
N-(2-Hydroxyethyl)-phenylacetamide	$C_{10}H_{13}NO_2$	A	61 - 62	7.81	7.70
N-Allylphenylacetamide	$C_{11}H_{13}NO$	B	53 - 55	8.00	7.92
N-(2-Methoxyethyl)-phenylacetamide	$C_{11}H_{15}NO_2$	"	Oil	7.25	6.93
N-(1-Hydroxyisopropyl)-phenylacetamide	$C_{11}H_{15}NO_2$	A	80 - 81	7.25	7.20
N-(2-Hydroxypropyl)-phenylacetamide	$C_{11}H_{15}NO_2$	"	49 - 52	7.25	7.18
N-(1,3-Dihydroxyisopropyl)-phenylacetamide	$C_{11}H_{15}NO_3$	"	129 - 132	6.69	6.89
N-(2,3-Dihydroxypropyl)-phenylacetamide	$C_{11}H_{15}NO_3$	C	38 - 40	6.69	6.11
N-(3-Aminopropyl)-phenylacetamide	$C_{11}H_{16}NO$	D	97 - 100	16.08	15.63
N-Crotylphenylacetamide	$C_{12}H_{16}NO$	B	57 - 59	*	
N-(β -Methylallyl)-phenylacetamide	$C_{12}H_{16}NO$	E	46.5- 48		
α -Phenylacetylaminic- <i>n</i> -butyric acid	$C_{12}H_{16}NO_3$	B	124 - 126	6.33	6.44
N-(2-Acetoxyethyl)-phenylacetamide	$C_{12}H_{16}NO_3$	F	75 - 78	6.33	6.30
N-(2-Hydroxyethyl)-phenacetamide	$C_{12}H_{16}N_2O_2$	A	143 - 144	11.86	12.01
N-(2-Hydroxyethyl-2-methylpropyl)-phenylacetamide	$C_{13}H_{17}NO_2$	"	67 - 69	6.27	6.75
N-(1-Hydroxy-2-butyl)-phenylacetamide	$C_{13}H_{17}NO_2$	"	54 - 56	6.76	6.82
N-(2-Hydroxybutyl)-phenylacetamide	$C_{13}H_{17}NO_2$	"	57 - 59	6.76	6.81
N-(1,1-Dimethyl-2-hydroxyethyl)-phenylacetamide	$C_{13}H_{17}NO_2$	"	Oil	6.76	6.68
N-(2-Hydroxyethyl)- γ -phenylbutyramide	$C_{13}H_{17}NO_2$	"	"	6.76	7.40
N-Ethyl-N-(2-hydroxyethyl)-phenylacetamide	$C_{13}H_{17}NO_2$	"	72 - 73	6.76	6.82
N-(2-Ethoxyethyl)-phenylacetamide	$C_{13}H_{17}NO_2$	B	47 - 48	6.76	6.79
N,N-Di-(2-hydroxyethyl)-phenylacetamide	$C_{13}H_{17}NO_3$	A	Oil	6.27	6.75
N-(2-Aminoethyl)- γ -phenylbutyramide hydrochloride	$C_{13}H_{18}NO_2 \cdot HCl$	G	95 - 98	11.57	11.78

TABLE IV—*Concluded*

Compound	Empirical formula	Method of preparation	M.p.	Nitrogen	
				Calculated	Found
			°C.	per cent	per cent
α -(Phenylacetyl-amino)- β,β -dimethylacrylic acid	$C_{13}H_{15}NO_3$	H	176 –177	6.01	5.99
N-(γ,γ -Dimethylallyl)-phenylacetamide	$C_{15}H_{17}NO$	E	66 – 68		
N-(2-Pentenyl)-phenylacetamide	$C_{15}H_{17}NO$	B	65 – 66	6.89	7.39
N-Phenylacetyl-DL-norvaline	$C_{13}H_{17}NO_3$	"	136 –138	5.96	5.95
N-Phenylacetyl-DL-penicillamine	$C_{13}H_{17}NO_3S$	I	125 –127	5.24	5.21
N-Phenylacetyl-L-penicillamine	$C_{13}H_{17}NO_3S$	J	132 –134	5.24	5.24
N-Phenylacetyl-D-penicillamine	$C_{13}H_{17}NO_3S$	"	132 –134	5.24	5.20
N-Phenylacetyl-DL- β -hydroxyvaline	$C_{13}H_{17}NO_4$	K	119 –121	5.57	5.53
N-Phenylacetyl-DL-valine amide	$C_{13}H_{15}N_2O_2$	A	195 –197	11.97	11.92
N-Phenylacetyl-DL-N-methylvaline	$C_{14}H_{19}NO_3$	B	104 –105	5.62	5.24
N-Phenylacetyl-DL-valine methyl ester	$C_{14}H_{19}NO_3$	L	52 – 53	5.62	5.73
N-Phenylacetylglucosamine	$C_{14}H_{19}NO_6$	K	214 –217	4.71	4.83
N-(2-Ethyl-2-hydroxybutyl)-phenylacetamide	$C_{14}H_{21}NO_3$	B	85 – 86	5.95	5.99
N-(2-Butyroxethyl)-phenylacetamide	$C_{14}H_{21}NO_3$	F	45 – 48	5.62	5.60
N-Phenylacetyl-DL- β,β -diethylalanine	$C_{16}H_{21}NO_3$	B	98 –100	5.3	5.6
N-(γ -Phenylbutyryl)-DL-valine	$C_{16}H_{21}NO_3$	"	110 –112	5.32	5.28
N-(2-Hydroxyethyl)- α -phenylacetylaminoisovaleramide	$C_{16}H_{23}N_3O_3$	M	Oil	10.07	10.04
N-(2-Isocaproxyethyl)-phenylacetamide	$C_{16}H_{23}NO_3$	F	"	5.06	5.05
N-(2-Phenylethyl)-phenylacetamide	$C_{16}H_{17}NO$	A	83 – 86	5.86	5.84
N-(2-Hydroxy-2-phenylethyl)-phenylacetamide	$C_{16}H_{17}NO_2$	B	94 – 96	5.49	5.69
Butyl DL- α -phenylacetylaminon-valerate	$C_{17}H_{25}NO_3$	N	27 – 28	4.81	4.75

* $C_{13}H_{15}NO$, calculated, C 76.15, H 7.99; found, C 76.09, H 7.61.

times from benzene-petroleum ether to yield 7.5 gm. of N-(β -methylallyl)-phenylacetamide.

Analysis— $C_{13}H_{15}NO$. Calculated. C 76.15, H 7.99
 Found. " 76.47, " 8.25

N-(γ,γ -Dimethylallyl)-phenylacetamide was prepared in the same manner with γ,γ -dimethylallyl chloride.

Analysis— $C_{13}H_{17}NO$. Calculated. C 76.81, H 8.43
Found. " 77.01, " 8.74

Method F—A solution of N-(2-hydroxyethyl)-phenylacetamide (Table IV) in dry pyridine was treated with 1 equivalent of the appropriate acid anhydride. The mixture was heated at 60° for several hours and then poured into ice water. The resulting oils, which soon solidified, were recrystallized from alcohol-water.

Method G—Ethyl γ -phenylbutyrate was heated with excess ethylenediamine for several hours. The volatile constituents of the mixture were removed by heating *in vacuo*. The residue was dissolved in an alcohol-ether mixture, and the solution was treated with dry hydrogen chloride to precipitate N-(2-aminoethyl)- γ -phenylbutyramide hydrochloride.

Method H—A solution of 1.5 gm. of N-phenylacetyl-DL- β -hydroxyvaline (Table IV) in 5 ml. of acetic anhydride was heated at 70° for 1 hour, and then evaporated to dryness *in vacuo*. To the residue were added 5 ml. of water and 6 ml. of acetone. The solution was heated under a reflux for 1 hour. After removal of the acetone by distillation, a crystalline product separated. The material was collected on a filter, washed with 1:1 chloroform-petroleum ether, and recrystallized from 15 ml. of acetone to yield 1.0 gm. of α -phenylacetyl-amino- β,β -dimethylacrylic acid.

Method I—N-Phenylacetyl-S-benzyl-DL-penicillamine was prepared from phenylacetyl chloride and S-benzylpenicillamine by the Schotten-Baumann method. It was recrystallized from ethylene dichloride, m.p. 66–68°.

Analysis— $C_{20}H_{27}NO_2S$. Calculated, N 3.9; found, N 3.7

A solution of 5 gm. of the N-phenylacetyl-S-benzyl-DL-penicillamine in 200 ml. of liquid ammonia was treated with sodium in small pieces until a blue color persisted. The excess sodium was neutralized with a little ammonium chloride and the ammonia was allowed to evaporate. The residue was dissolved in water and warmed under a vacuum to remove any residual ammonia. The N-phenylacetyl-DL-penicillamine was precipitated with hydrochloric acid and recrystallized from ethyl acetate-petroleum ether.

Method J—A solution of 21.0 gm. (0.059 mole) of N-phenylacetyl-S-benzyl-DL-penicillamine in 50 ml. of methanol was treated with a solution containing 26.9 gm. (0.059 mole) of brucine. The crystals which separated on standing were collected, washed with a little absolute alcohol, and air-dried. They weighed 20.5 gm., m.p. 108–109°. After three recrystallizations from absolute alcohol, there were obtained 15.9 gm. of crystals of

constant rotation, m.p. 117–119°, $[\alpha]_D^{20} = -19.0^\circ$ (in 1 per cent absolute alcohol solution). This salt, 14.9 gm., yielded 6.4 gm. of crude N-phenylacetyl-S-benzyl-L-penicillamine which after three recrystallizations from 3:1 water-alcohol gave 5.5 gm. of pure acid of constant rotation; m.p. 141–142°, $[\alpha]_D^{28.2} = -7.5^\circ$ (in 2 per cent absolute alcohol solution).

The filtrate from the first crop of the brucine salt was evaporated *in vacuo* to dryness. 200 ml. of water were added and the crystals, 20 gm., were collected on a filter and recrystallized from 4:1 water-alcohol to yield 15.8 gm. of salt of constant rotation, m.p. 108–111°, $[\alpha]_D^{27.5} = -3.75^\circ$ (in 4 per cent absolute alcohol solution). From 14.8 gm. of this salt there were obtained 5.6 gm. of N-phenylacetyl-S-benzyl-D-penicillamine, m.p. 141–142°, $[\alpha]_D^{28.2} = +7.5^\circ$ (in 2 per cent absolute alcohol solution).

The N-phenylacetyl-S-benzylpenicillamines were converted to the respective N-phenylacetyl-L-penicillamine and N-phenylacetyl-D-penicillamine as described under Method G, above.

Method K—These compounds were prepared by the Schotten-Baumann method with sodium bicarbonate.

Method L—Phenylacetyl-DL-valine was esterified with diazomethane in ether. The ester was recrystallized from ethyl acetate-petroleum ether.

Method M—Phenylacetylvaline methyl ester was dissolved in 10 parts of methanol saturated at 0° with ammonia. After standing at room temperature for 4 days the solution was chilled. The crystalline amide was collected and recrystallized from alcohol-water.

Method N—10 gm. of DL- α -amino-*n*-valeric acid were esterified with 200 ml. of dry *n*-butanol saturated with hydrogen chloride. The resulting ester hydrochloride was phenylacetylated with 11.5 ml. of phenylacetyl chloride and dilute Na_2CO_3 solution. The resulting oil was crystallized by cooling in a dry ice bath. Recrystallization from petroleum ether yielded 2.7 gm. of product.

Resolution of Phenylacetyl-DL-valine

Phenylacetyl-DL-valine, 23.5 gm. (0.1 mole), was dissolved in 50 ml. of methanol and a solution of 46.7 gm. (0.1 mole) of brucine in 100 ml. of methanol was added slowly to it, with constant stirring. The solution was allowed to stand in a refrigerator overnight and no crystallization of the salt occurred. Most of the solvent was evaporated under reduced pressure and the oily mass left was dissolved in 400 to 450 ml. of hot water. After cooling, a crop of colorless needles was obtained which weighed 55.0 gm. and melted at 105–108°.

The crystals were recrystallized five times from water; m.p. 105°, $[\alpha]_D^{30.5} = -9.0^\circ$ (2 per cent absolute ethanol solution). The yield was 20.1 gm.

14 gm. of this salt were suspended in 100 ml. of water. Excess of dilute sodium hydroxide solution was added, and the precipitate of brucine was filtered off and washed several times with water. The filtrate was made acid by adding an excess of concentrated hydrochloric acid. The precipitate was filtered and washed twice with water and air-dried overnight; yield 4.7 gm., m.p. 139–140°. Two recrystallizations from alcohol-water (1:3) gave the pure product, 3.2 gm., m.p. 139–140°, $[\alpha]_D^{20.2} = +9.6^\circ$, in 4 per cent absolute ethanol. It gave no depression in a mixed melting point determination with an authentic sample of phenylacetyl-D-valine.

The filtrate from the first crop of brucine salt was evaporated to dryness under reduced pressure. The residue was dissolved in 100 ml. of water and decolorized with a little charcoal. It was evaporated again to dryness under reduced pressure, 100 ml. of ether were added, and the solid collected. It weighed 15.0 gm., m.p. 105–108°. Four recrystallizations from water gave a product of constant rotation; yield 6.0 gm., m.p. 106–107°, $[\alpha]_D^{20.5} = -19.6^\circ$ (2 per cent absolute ethanol solution).

The salt was converted into the free acid in the same manner as the isomeric compound. From 6.0 gm. of the salt, 1.7 gm. of crude phenylacetyl-L-valine were obtained, m.p. 130–132°. After four recrystallizations from alcohol-water (1:3), 0.42 gm. of a product of constant rotation was obtained; m.p. 137–138°, $[\alpha]_D^{28.7} = -9.0^\circ$ (in 4 per cent absolute ethanol solution).

We are happy to acknowledge helpful discussions and suggestions contributed by Professor H. E. Carter of the University of Illinois.⁴ The authors also express their gratitude to Dr. G. H. A. Clowes and Dr. E. C. Kleiderer for their interest in this work. The microanalyses were performed by W. L. Brown and H. L. Hunter.

SUMMARY

1. Methods have been described for evaluating compounds as precursors for benzylpenicillin.

2. Addition to media of certain compounds containing the phenylacetyl group or of certain compounds which may be converted biologically to contain this group has resulted in increased production of benzylpenicillin.

3. Benzylpenicillin has been isolated after use of N-(2-hydroxyethyl)- γ -phenylbutyramide as a precursor.

4. Numerous compounds which have been tested as benzylpenicillin precursors (*cf.* (2)) have been prepared.

⁴ Valuable technical assistance has been given by Charlotte Harris, John O'Brien, and Dorothea Huff.

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BIOSYNTHESIS OF PENICILLINS

II. UTILIZATION OF DEUTEROPHENYLACETYL-N¹⁵-DL-VALINE IN PENICILLIN BIOSYNTHESIS*

By OTTO K. BEHRENS, JOSEPH CORSE,† REUBEN G. JONES,
E. C. KLEIDERER, QUENTIN F. SOPER, AND
F. R. VAN ABELE

(From the Lilly Research Laboratories, Indianapolis)

L. M. LARSON AND J. Č. SYLVESTER

(From the Abbott Laboratories, North Chicago)•

WILLIAM J. HAINES

(From The Upjohn Company, Kalamazoo)

AND HERBERT E. CARTER

*(From the Division of Biochemistry, Noyes Laboratory of Chemistry,
University of Illinois, Urbana)*

(Received for publication, February 28, 1948)

In Paper I in this series (2) the stimulation of penicillin production by certain compounds containing the phenylacetyl group was reported. It was realized that a stimulation of yield of this biological product following addition of a given substance to the medium may result from any one of several possible metabolic mechanisms. The stimulating substance may act as a vitamin or growth promoter, as a building block to be incorporated into the organism, or it may be required to satisfy any one of several other types of metabolic requirements. Thus, the early observations of increased yields were subject to varied interpretations, and there was need for proof of the direct utilization of the substances in penicillin biosynthesis.

To provide such proof the preparation and use as a precursor of deuterophenylacetyl-N¹⁵-valine was proposed (L, 13, 17).¹ Considerations in the choice of this particular compound included the knowledge that considerable specificity was exhibited by both acyl and amide portions of the molecule, and the fact that the amide portion of the molecule contained a carbon skeleton similar to that in penicillamine. The deuterophenylacetyl-N¹⁵-valine was prepared in the Lilly laboratories (L, 23, 8) from deuterophenyl-

* The work reported here in detail is briefly discussed in the monograph on penicillin (1).

† Present address, University of California, Los Angeles, California.

¹ The references give the number and page of reports made to the Office of Scientific Research and Development.

acetic acid² and N^{15} -valine (L, 22, 1). The cultural work and preliminary purification were conducted in the Abbott laboratories and final purification and crystallization of the penicillin was carried out in the Upjohn laboratories (U, 22, 8). The isotopic determinations were performed by Dr. Rittenberg. By means of the deuterium analysis, it was shown that 92.5 per cent of the penicillin obtained was derived from the precursor. In sharp contrast the N^{15} content of the isolated penicillin was only 2.69 per cent of the value expected if the phenylacetylvaline had supplied 1 nitrogen atom to the penicillin. These results demonstrated that the phenylacetyl moiety appeared in the penicillin formed, but that the valine portion of the precursor was not directly utilized in penicillin formation. The rôle of the amide portion of such precursors thus remains undefined.

EXPERIMENTAL

N^{15} -DL-Valine (L, 22, 1)— N^{15} -DL-Valine was prepared by the method of Knoop (3) as adapted for isotopic synthesis by Schoenheimer and Ratner (4).

Ammonium nitrate prepared by the Eastman Kodak Company, containing about 32 per cent of N^{15} in the ammonium moiety, was used. Ammonia was generated from 4.0 gm. (0.05 mole) of this ammonium nitrate and was absorbed in 35 ml. of ethanol containing 4 gm. of palladium black and cooled in a dry ice bath. After absorption of the ammonia was complete, 2.90 gm. (0.025 mole) of α -oxoisovaleric acid (5) in 35 ml. of water were added.

The mixture of isotopic ammonia and α -oxoisovaleric acid was hydrogenated under a pressure of 2 atmospheres. Almost the theoretical amount of hydrogen was absorbed. The excess $N^{15}H_3$ was recovered as directed by Schoenheimer and Ratner (4).

The contents of the hydrogenation vessel were treated with hot water, filtered, and evaporated to dryness *in vacuo*. Ethanol was added and the evaporation was repeated. The resulting white, microcrystalline powder was suspended in ethanol, collected on a filter, and washed with ether. After drying, the product weighed 1.26 gm. (43 per cent yield).

Analysis— $C_6H_{11}NO_2$. Calculated,³ N 12.19; found, N 12.00

Deuterophenylacetyl- N^{15} -DL-valine (L, 23, 8)—A mixture of 2.90 gm. (0.021 mole) of deuterophenylacetic acid (in which 41 per cent of the hydrogen atoms in the benzene nucleus had been replaced with deuterium) and 5 ml. of pure thionyl chloride was allowed to stand at room temperature for 18 hours. The excess thionyl chloride, the hydrogen chloride, and the sulfur di-

² Sample furnished by Dr. David Rittenberg, College of Physicians and Surgeons, Columbia University.

³ The calculated value is based on 32 per cent N^{15} .

oxide were evaporated at room temperature under a pressure of 5 to 10 mm. The phenylacetyl chloride was dissolved in 20 ml. of dry benzene. This benzene solution was added dropwise over a period of 10 minutes to a rapidly stirred solution of 2.60 gm. (0.022 mole) of the N^{15} -DL-valine in 25 ml. of 2.6 N sodium hydroxide solution, cooled in an ice-salt bath. After the addition of the phenylacetyl chloride was complete, the cooling bath was removed, and the stirring was continued for 1 hour at room temperature. The benzene layer was separated and the aqueous layer was washed with 50 ml. of ether. The combined benzene and ether solutions were washed with two 15 ml. portions of water. The total water solution was acidified with hydrochloric acid. After standing, the precipitated oil crystallized. The product was collected, washed with a little water, dried, and then washed with 50 ml. of hot petroleum ether (b.p. 60–68°). The yield of white crystalline solid was 4.14 gm. (84 per cent).

Analysis— $C_{11}H_{17}NO_2$. Calculated, ^{4} N 6.00; found, N 5.86

Benzylpenicillin from Deuterophenylacetyl- N^{15} -DL-valine—The isotopic precursor was sent to the Abbott laboratories where it was added at a level of 188 mg. per liter to a corn steep medium inoculated with *Penicillium notatum*, strain NRRL 1976.

Approximately 5 liters of filtered broth assaying 95 units per ml. were chilled to 5°, acidified to pH 2.0, and extracted with amyl acetate. The amyl acetate solution was extracted with 3 per cent phosphate buffer solution at pH 7.0. The phosphate buffer solution was chilled to 5°, acidified to pH 2.0, and extracted with chloroform. The penicillin in the chloroform solution was converted to sodium salt by stirring with water and adding sodium hydroxide dropwise until the pH reached 7.0. The aqueous solution was dried from the frozen state.

This crude sodium salt was sent to the Upjohn laboratories for the isolation of the crystalline penicillin (U, 22, 8). The sodium penicillin (352,000 units, assaying 234 units per mg.) was further purified by use of an ether-silica-phosphate buffer (pH 6.2) chromatographic column (2). The four bands of activity that were noted accounted for 99 per cent of the units applied to the column. The benzylpenicillin band containing 191,000 units was converted to the sodium salt, yielding 175,000 units of material which assayed 790 units per mg. It seemed probable that the yield of crystalline product from this material would be insufficient for adequate isotopic analysis. Hence, 100 mg. (an equal amount in terms of units of antibiotic activity) of analytically pure sodium benzylpenicillin were added. The combined material (321 mg., assaying 1150 units per mg.) was further puri-

^{4} The content of D and N^{15} being taken into account.

fied over a chloroform-silica-phosphate buffer (pH 6.2) column. All the recovered activity, representing 91 per cent of the applied penicillin, was in a single band. This material was converted to the sodium salt and was dried from the frozen state, yielding 230,000 units assaying 1590 units per mg. The product (145 mg.) was treated with dry acetone, in which it first dissolved, then reprecipitated, yielding 137 mg. of powder. The powder was dissolved in 0.65 ml. of 90 per cent acetone. Addition of 1.1 ml. of dry acetone to the solution yielded a first crop of crystals weighing 70 mg. This material, which consisted of typical benzylpenicillin platelets, was used for isotopic analysis.

Analysis—C₁₆H₁₇N₂O₄SNa.

Calculated. C 53.92, H 4.81, N 7.83

Found. " 54.02, " 5.10, " 8.02

An additional 38.4 mg. of crystalline material were obtained from the mother liquors of the first crystallization. Thus a total of 108.4 mg. of crystallized material was recovered from the acetone-insoluble residue of 137 mg.

Deuterium and N¹⁵ determinations on the penicillin and on a sample of the precursor were performed by Dr. David Rittenberg. The original phenylacetic acid used in the preparation of the precursor contained 26.7 atom per cent excess deuterium (L, 28, 3). The phenylacetylvaline contained 32.7 atom per cent excess N¹⁵. The crystalline sodium benzylpenicillin isolated (which had been diluted during processing with an equal amount of non-isotopic benzylpenicillin) contained 5.81 atom per cent excess deuterium and 0.220 atom per cent excess N¹⁵.

Calculation shows that 92.5 per cent of the benzylpenicillin isolated was derived from the precursor as indicated by the deuterium analysis.

$$\begin{aligned} 0.0581 \times 17 \times 2 &= 1.975 \text{ atoms of D in the isolated penicillin} \\ 0.267 \times 8 &= 2.136 \text{ atoms of D in the original phenylacetic acid} \\ 1.975/2.136 &= 0.925 \end{aligned}$$

In sharp contrast, only 2.69 per cent of the penicillin isolated was derived from the part of the precursor containing N¹⁵.

$$\begin{aligned} 0.0022 \times 2 \times 2 &= 0.0088 \text{ atom of N}^{15} \text{ per molecule of isolated penicillin} \\ 0.327 \times 1 &= 0.327 \text{ atom of N}^{15} \text{ per molecule of phenylacetylvaline} \\ 0.0088/0.327 &= 0.0269 \end{aligned}$$

SUMMARY

The preparation of deuterophenylacetyl-N¹⁵-DL-valine is described.

Benzylpenicillin was isolated following use of this as a precursor. Deuterium analyses demonstrated that the phenylacetyl portion of the precursor

was incorporated directly into the penicillin. In contrast, very little N¹⁵ was found in the penicillin product. Therefore, the rôle of the amide portion is still unknown.

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BIOSYNTHESIS OF PENICILLINS

III. PREPARATION AND EVALUATION OF PRECURSORS FOR NEW PENICILLINS

By OTTO K. BEHRENS, JOSEPH CORSE,* DOROTHEA E. HUFF,
REUBEN G. JONES, QUENTIN F. SOPER, AND
CALVERT W. WHITEHEAD

(From the Lilly Research Laboratories, Indianapolis)

(Received for publication, February 28, 1948)

Papers I and II in this series (1, 2) have presented data concerning the stimulation of biological production of benzylpenicillin by use of appropriate precursors, with proof that the acyl portion of these compounds (or part of it) appears in the penicillin. At a very early stage of this work serious consideration was given to the possibility of using a similar method to influence the mold to form new penicillins. Preparation of a considerable series of compounds containing acyl groups other than those appearing in the natural penicillins was initiated. It soon became apparent that the mold possessed an amazing ability to utilize a wide variety of acyl groups, with the concomitant formation of new penicillins. These new penicillins were often produced in good yields (60 to 100 per cent of the total penicillin formed) and in a number of cases have been isolated in analytically pure, crystalline form.

In spite of the fact that it was already known that the mold could produce several different natural penicillins, serious doubt had existed concerning the possibility of obtaining new penicillins. It is well known that most enzyme systems display a remarkable specificity. In many cases this specificity is such that only a single substrate is amenable to transformation, and only in a few cases do enzymes demonstrably exert wide multiplicity of action. Thus, it seemed probable that the several natural penicillins were formed respectively by separate enzyme systems or by one enzyme able to synthesize a limited number of penicillins.

Several criteria have been used in the evaluation of compounds as precursors. It is obvious that final proof of the utilization of compounds must reside in isolation of analytically pure penicillins in which the identity of the acyl group may be established. However, in view of the considerable effort involved in such isolations, particularly with new compounds differing from one another in properties, other more simple criteria were found helpful.

The simplest of these was the stimulation test. This test was first applied in the evaluation of precursors for benzylpenicillin (1), and its general

* Present address, University of California, Los Angeles, California.

validity for that purpose was demonstrated in numerous experiments. The stimulation tests with strain NRRL 1976 have been supplemented in many instances with tests in which the higher yielding *Penicillium chrysogenum*, Q-176, was used.

A stimulation in yield was observed with numerous compounds. Data obtained before the conclusion of the penicillin contract are contained in the monograph on penicillins (3). Subsequent experiments are reported in this (*cf.* Table II) and succeeding articles in this series. The view has been generally justified that a stimulation may be interpreted as indicative of the utilization of the precursor. A few exceptions have been observed. In these instances, the increased yield must be explained on the basis of a function of the added compound other than that of precursor (*cf.* (2)).

In contrast, an absence of stimulation or a diminution of total yield cannot be interpreted as lack of utilization of a compound, although this is often the case. The stimulation of penicillin yield observed with many compounds may be explained through an increase in the total molar production of penicillins, augmented in some cases by the higher activity of the new penicillin. It is obvious that no significant stimulation will be observed if the new penicillin formed is only a small portion of the total produced, or if a new penicillin formed in good yield is less active on a molar basis than the aggregate of natural penicillins produced without use of a precursor. For these reasons, evaluation of new compounds as possible precursors requires criteria other than that of yield.

Differential assays¹ on the broth obtained with a test compound have often been of assistance in determining whether a compound is utilized. The differential assay values are characteristic for the individual penicillins, and those for the natural penicillins vary from about 0.35 for *n*-heptylpenicillin to approximately 1.3 for *p*-hydroxybenzylpenicillin. With a fixed set of cultural conditions the proportions of the various natural penicillins formed by a given mold strain remain reasonably constant. Thus, comparison of the differential assay value on a control broth with that on a broth obtained in the presence of a test compound may indicate whether the proportion of the penicillins has been altered. Any such alteration may be interpreted as indicating the presence of a new penicillin. In some cases the new penicillin may have a differential assay value similar to that of the mixture in the control broth. For this reason, a failure to note a difference must not be considered as proof of lack of utilization of the test compound.

¹ The differential assay is the ratio of antibacterial activity for *Bacillus subtilis* to *Staphylococcus aureus* compared with that for pure benzylpenicillin, which is defined as 1.00. A description of the method of determination is given in the monograph (3). We acknowledge with thanks the numerous assays performed by Dr. J. M. McGuire of these laboratories.

An additional criterion has been of value in supplementing the stimulation tests and differential assay determinations. During chromatographic purification and separation of penicillins (*cf.* (1)) it is well known that the various natural penicillins are found in differing positions on the column and in the filtrates. With a silica column, phosphate buffer (pH 6.2), and ether, *p*-hydroxybenzylpenicillin is found at the top, *n*-heptylpenicillin is found in the filtrates, and other natural penicillins are found in intermediate locations. These differences reflect variations in the distribution coefficient of the various penicillins between the aqueous buffer and the organic solvent.

After chromatographing the penicillin from a broth obtained in the presence of a precursor, it is often found that the distribution of the penicillins is markedly different from that observed with a control broth. In many

TABLE I

Comparison of Chromatographic Columns of Penicillin Obtained with and without Precursor

A = broth with no precursor; B = broth with N-(2-hydroxyethyl)-allylmercaptoacetamide. The figures give the units in each fraction.

Fraction	A	B	Fraction	A	B
S-1	None	1,000,000	S-9	700,000	None
S-2	"	3,600,000	S-10	600,000	"
S-3	<300,000	7,600,000	F-1	<300,000	"
S-4	<300,000	9,900,000	F-2	600,000	"
S-5	700,000	7,200,000	F-3	1,600,000	<300,000
S-6	1,200,000	1,000,000	F-4	3,600,000	None
S-7	900,000	<300,000	F-5	2,100,000	"
S-8	1,100,000	None			

cases it is observed in a single rather sharp band. This phenomenon is illustrated in Table I. In the absence of a precursor, the penicillin was distributed over most of the column, and a large fraction of *n*-heptylpenicillin was found in the filtrate fractions F-3, F-4, and F-5. In contrast, the penicillin formed in the presence of N-(2-hydroxyethyl)-allylmercaptoacetamide was found predominantly on the silica column in fractions S-3, S-4, and S-5 and very little activity was present in the filtrate fractions.

Contrasts such as that noted above were often observed during the purification of new penicillins. In most cases, however, small differences would be of doubtful significance because of the difficulties in preparing successive columns in exactly the same manner. Recently a new technique has been made available through the work of Craig and coworkers (4) that makes possible an accurate comparison of the distribution of compounds

between two solvents. We have utilized the Craig separation as an exploratory tool in the following manner. Control and experimental broths were prepared under similar cultural conditions. The penicillin was recovered from the respective lots as described in the experimental section. The penicillin was dried from the frozen state and was subjected to the Craig separation technique. Approximately 20,000 units were used with ether and 2 M phosphate buffer, pH 4.8.

Fig. 1 illustrates the distribution curves obtained from penicillin produced by *Penicillium chrysogenum*, Q-176, with (a) control (no precursor), (b) *p*-mercaptophenylmercaptoacetic acid, and (c) *p*-(*N*-*p*'-arsonophenylsulfamyl)-phenylmercaptoacetic acid. It will be noted that the curve obtained with *p*-(*N*-*p*'-arsonophenylsulfamyl)-phenylmercaptoacetic acid is similar to that obtained with the control broth, and that differential assays of comparable samples are similar. It is concluded that this compound is not effectively utilized by this strain. It should be noted, of course, that the formation of a small amount of a new penicillin with a distribution coefficient such that it would be included in the large band in Tubes 7 to 15, and with a differential assay similar to the natural penicillins occurring in these tubes, would be difficult to detect. With *p*-mercaptophenylmercaptoacetic acid as precursor, the distribution curve is markedly different from that in the control. The large centrally located band is markedly diminished and a considerable portion of the activity is found in Tubes 19 to 24. The differential assay values on these samples are higher than those obtained with a comparable control sample. Therefore, we may conclude that this compound serves as a precursor leading to production of a new penicillin with a differential assay value of 0.8 or higher.

Under our conditions of testing, the distribution of penicillin was similar to that observed with control cultures when β -(*p*-bromophenyl)-isovaleryl-DL-valine served as the precursor with *Penicillium notatum* NRRL 1976 or when benzylsuccinic acid, N-(*N*'-*p*-chlorobenzoyl-DL-alanyl)-DL-valine, *p*-chlorocarbobenzoxyglycine, β -(*p*-nitrophenyl)-isovaleryl-DL-valine, N-(2-hydroxyethyl)-vinylacetamide, N-(2-hydroxyethyl)- β -hydroxy- γ , γ , γ -trifluorobutyramide, carbobenzoxyglycine, N-(2-hydroxyethyl)-phenylsulfonfylacetamide, or N-(β -phenylmercaptopropionyl)-DL-valine was used as precursor with *Penicillium chrysogenum* Q-176.

Additional information concerning such distribution studies will be reported in subsequent articles, along with the preparation of the respective compounds used as precursors.

In a few cases efforts have been made to determine utilization of precursors by isolation, even though the stimulation data and other preliminary methods of evaluation were not encouraging. These isolation efforts have demonstrated that the following compounds were not effectively utilized

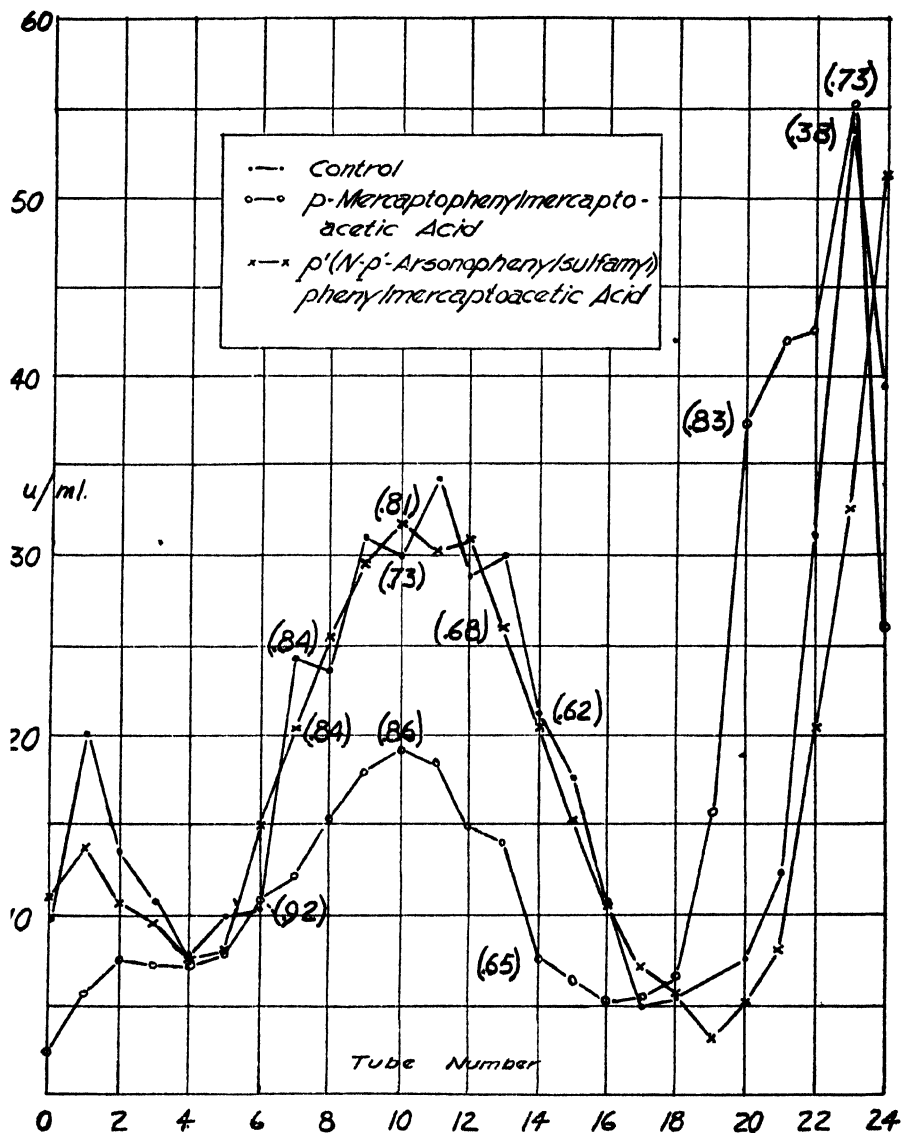


FIG. 1. "Craig machine" distribution curves. The numbers in parentheses represent differential assay values on the samples indicated.

under our cultural conditions: tryparsamide, β, β -diphenylpropionic acid, *N*-allyl- β -chloropropionamide, and 2-thiophenecarboxylic acid (and several derivatives).

Stimulation data for numerous compounds which were tested as possible

new penicillin precursors have been presented (3). The preparation of most of these compounds has not been described previously. The preparation and some properties of acids and certain derivatives of the following types are included in this paper: aryl carboxylic acids, α -substituted phenylacetic acids, aliphatic acids, aryl aliphatic (other than acetic) acids, and a miscellaneous unclassified group. Subsequent papers in this series will describe the preparation of ring-substituted phenylacetic acids, substituted oxy- and mercaptoacetic acids, and polycyclic and heterocyclic acetic acids and their derivatives.

EXPERIMENTAL

Stimulation Tests—The tests were performed with *Penicillium notatum* NRRL 1976 by the "low" (L) and "high" (H) methods described in Paper I in this series (1). Additional tests were performed with *Penicillium chrysogenum* Q-176 with a medium containing 3 per cent corn steep solids, 2.5 per cent lactose, and 0.5 per cent calcium carbonate or with a synthetic medium containing 20 gm. of lactose, 10 gm. of glucose, 4 gm. of ammonium acetate, 5 gm. of ammonium lactate, 3 gm. of monopotassium phosphate, 0.25 gm. of magnesium sulfate, 0.02 gm. of zinc sulfate, 0.02 gm. of ferrous sulfate, 0.02 gm. of manganous sulfate, 0.5 gm. of sodium sulfate, and 0.0025 gm. of cupric sulfate per liter plus sodium hydroxide to pH 6.2. 80 ml. of the synthetic medium were placed in each 500 ml. Erlenmeyer flask, and after sterilization, 10 ml. of a 2 day vegetative inoculum prepared in corn steep medium were added.

"Craig Machine" Distribution Curves—A control broth (no precursor) and several experimental broths were prepared under similar cultural conditions. The broths were filtered to remove mycelial growth, chilled to 0–5°, and, following acidification to pH 2.2 with 10 per cent phosphoric acid, were extracted with an equal volume of cold amyl acetate. The amyl acetate layer was separated, and the sodium salt of the penicillin was formed by addition of 0.1 N sodium hydroxide solution with stirring to pH 7.5. The aqueous solution was separated and the amyl acetate was washed with a small portion of water with addition of small quantities of alkali as required to maintain the proper pH. The combined aqueous solutions were adjusted to pH 6.5 with dilute phosphoric acid and were dried from the frozen state.

Approximately 20,000 units of crude penicillin obtained in this manner were carried through the separation procedure with 2 M phosphate buffer, pH 4.8, and ether in a manner similar to that described by Craig, Hogeboom, du Vigneaud, and Carpenter (5). The distribution of the penicillins over the twenty-five tubes was determined by microbiological assay of the penicillin content of each tube. The ether-phosphate mixture in each tube

was withdrawn and a predetermined quantity of sodium hydroxide solution was added to adjust the pH to 7.0 to 7.5. The resulting mixture was shaken and the phosphate layer was separated for penicillin determination.

Attempted Isolation of Penicillins

1. N-Allyl- β -chloropropionamide (122 mg. per liter) served as the precursor with *Penicillium notatum* NRRL 1976. The penicillin was produced and purified in a manner similar to that described in Paper I (1) for benzylpenicillin from N-(2-hydroxyethyl)- γ -phenylbutyramide. All of the most active fractions on the ether chromatographic column were combined. The chloroform column yielded a single, rather sharp band. The penicillin was recovered as the sodium salt, which was dried from the frozen state. Efforts to crystallize the dark yellow material failed. It assayed 900 units per mg. and gave a differential assay value of 0.64.

Analysis— $C_{11}H_{14}ClN_2O_4SNa$. Calculated, Cl 10.8; found, Cl 0.15

2. β,β -Diphenylpropionic acid (181 mg. per liter) was used as the precursor with strain X-1612. A large portion of the activity recovered from the ether column was found in a broad band near the center of the column. This material was further purified by use of a chloroform column, and the recovered material was dried as the sodium salt. Treatment of the yellow powder with absolute acetone yielded a crystalline product. Some color was removed by washing with acetone. After one precipitation from 96 per cent aqueous acetone by addition of 5 parts of anhydrous acetone, the penicillin assayed 1700 units per mg. and gave a differential assay value of 0.84. The ultraviolet absorption spectrum² demonstrated the absence of the diphenylpropionic group and indicated that the penicillin probably contained an aliphatic acyl group.

3. Tryparsamide (440 mg. per liter) was added to a medium containing 3 per cent of corn steep solids, 2.5 per cent of lactose, and 0.5 per cent of calcium carbonate. 60 flasks, each containing 225 ml. of the medium, were inoculated with *Penicillium chrysogenum* Q-176. After 5 days the broth was harvested, and the penicillin was purified. The ether column appeared to contain three bands. The largest of these was further divided into two fractions on a chloroform column. No arsenic was detected in the lyophilized penicillin obtained from any of these fractions.

4. 2-Thiophenecarboxylic acid was provided by the simultaneous use of (a) the acid (26 mg. per liter), (b) the DL-valine derivative (46 mg. per liter), (c) the allylamide (33 mg. per liter), and (d) the ethanolamide (34 mg.

² The ultraviolet absorption data were obtained by Dr. W. W. Davis and T. V. Parke of these laboratories.

per liter). The fermentation was performed in a manner similar to that described for tryparsamide. The ether column showed considerable scattering. All fractions with appreciable activity were therefore recombined. The chloroform column indicated the presence of a single band. The recovered penicillin was dried as the sodium salt. Attempts to crystallize the material failed. The amorphous residue assayed 970 units per mg. and gave a differential assay value of 0.69.

Analysis— $C_{11}H_{13}N_2O_4S_2Na$. Calculated. N 8.04, S 18.41
Found. " 6.45, " 7.44

It was concluded that none of the above isolations yielded the desired penicillin. Apparently these compounds are not transformed into penicillins in appreciable quantities under the conditions described.

Preparation of Precursors—Table II lists some compounds which were tested as possible precursors for new penicillins, together with their stimulation values. If the compound is new, the method of preparation and some properties are also given.

Method A. Amides by Schotten-Baumann Method—The acid chloride was allowed to react with the amino compound, allylamine, ethanolamine, or DL-valine, in the usual manner in cold aqueous sodium hydroxide solution or in cold chloroform solution in the presence of pyridine or an excess of the amino compound. The amide was filtered or extracted from the aqueous phase. When chloroform was used, the solution was filtered free of solid and the amide was obtained by evaporation of the solvent. The majority of the amides were solids and were purified by recrystallization from ethylene dichloride or ethyl acetate. The oils were either distilled or separated from volatile impurities by warming under reduced pressure.

Method B. Amides from Esters—The ethyl or methyl ester was mixed with an excess of the amine, ethanolamine, allylamine, etc., and the mixture heated for 8 to 24 hours at 100–120°. The excess amine was removed by washing with dilute acid, or, when the amide was water-soluble, by warming *in vacuo*. The amide was further purified by crystallization from ethylene dichloride or ethyl acetate or by distillation.

Method C. Methyl β -S-Ethylmercaptopropionate—Ethyl mercaptan (40 gm.) was added in portions to 43 gm. of methyl acrylate and 2 drops of Triton B. The reaction was cooled when it became vigorous and the mixture then allowed to stand overnight; yield 61 gm., b.p. 109–113° at 55 mm.

Method D. N-(2-Hydroxyethyl)-allylsulfinylacetamide—A solution was prepared from 53.0 gm. of N-(2-hydroxyethyl)-allylmercaptoacetamide (50) and 100 ml. of acetone. To this solution were added, with stirring, 33.0 ml. of 30 per cent hydrogen peroxide solution. The resulting solution was

allowed to stand for 1 week and then the remaining solvent was evaporated in a current of air. The residue was dissolved in a boiling mixture of 250 ml. of ethyl acetate and 30 ml. of methanol. This solution was filtered hot and allowed to cool, yielding 34.8 gm. of solid; m.p. 77–79°. Recrystallization was effected from the mixture of solvents or ethyl acetate alone, m.p. 81.5–82°.

Method E. N-(2-Hydroxyethyl)-allylsulfonylacetamide—A solution of 44.5 gm. of *N*-(2-hydroxyethyl)-allylmercaptoacetamide (50), 1 liter of acetone, and 75 ml. of 30 per cent hydrogen peroxide solution was allowed to stand for 10 days. The volatile solvents were removed by a current of air, leaving an orange oil; yield 47.4 gm. after drying *in vacuo*.

Method F. Methyl β -Allyloxypropionate—The addition of 100 gm. of methyl acrylate to 200 ml. of allyl alcohol, in which were dissolved 5.3 gm. of sodium, produced a gelatinous precipitate. The mixture was heated on the steam bath for 1 hour and poured into water. The mixture was extracted several times with ether, the ether solution was dried, and the esters were distilled, b.p. 116–132° at 65 mm.; yield 50.3 gm., $n_D^{20.5} = 1.4312$, which was mainly the methyl ester.

Analysis— $C_7H_{12}O_3$. Calculated, C 58.31, H 8.39; found, C 59.87, H 8.61

The second fraction, b.p. 120–130° at 65 mm., yield 23.5 gm., $n_D^{20.5} = 1.4394$, was mainly the allyl ester.

Analysis— $C_8H_{14}O_3$. Calculated, C 63.51, H 8.29; found, C 63.06, H 8.60

Method G. N-(N'-p-Chlorobenzoyl-DL-alanyl)-DL-valine—DL-Alanyl-DL-valine (51) was treated with *p*-chlorobenzoyl chloride and 5 N sodium hydroxide solution according to the usual procedures. The yield was about 90 per cent of large, white needles which melted over a range of 172–205°. The product was insoluble in ether, chloroform, benzene, and petroleum ether. A low nitrogen analysis suggested the presence of *p*-chlorobenzoic acid as a contaminant. This acid was removed by washing with ether and recrystallizing the residue from dilute ethanol to give silver flakes, m.p. 204–206°.

Method H. Methyl β, β -Dimethyl- γ -pentynoate—A mixture of 130 gm. (1.55 mole) of freshly distilled dimethylacetylenecarbinol (52), 10 gm. of cupric oxide, 5 gm. of ammonium chloride, and 225 ml. of concentrated hydrochloric acid was vigorously shaken in a stoppered flask for $\frac{1}{2}$ hour while the temperature was maintained below 40°. Three such runs were combined. The non-aqueous layer was separated, washed with 200 ml. of concentrated hydrochloric acid and three 300 ml. portions of water, and dried over magnesium sulfate. Fractionation of the liquid yielded 160 gm. (34 per cent) of 3,3-dimethyl-3-chloro-1-propyne; b.p. 77–79° (53).

To a solution of 330 gm. (2.06 mole) of pure malonic ester in 1 liter of

TABLE II
Compounds Tested As New Penicillin Precursors

The table is arranged according to the empirical formula of the acids. The numbers under "Stimulation" represent the ratio of units in the test container to units in the control container. Most of the tests were performed with *Penicillium notatum* NRRL 1976. In a number of cases *Penicillium chrysogenum* Q-176 was also used with comparable results.

Name of acid and amide	Empirical formula	Method of preparation	M.p.	Analyses								Stimulation		
				Calculated				Found						
				C	H	N	per cent	C	H	N	per cent			
Cyanoacetic	$C_3H_3NO_2$	†	°C.											
2-Hydroxyethyl	$C_3H_5NO_2$	B	Oil	46.87	6.29			46.84	6.70					0.9
β-Chloropropionic	$C_3H_3ClO_2$	†												
Allyl	C_3H_5ClNO	A	39-40	48.82	6.83			48.98	6.67					1.0
β-Hydroxypropionic	$C_3H_5O_2$	(6)												
2-Hydroxyethyl	$C_3H_7NO_2$	B	73.5-75	45.10	8.35			45.14	7.97					1.0
γ-Trichlorobutyric	$C_3H_3Cl_3O_2$	(7)												
DL-Valine	$C_6H_{11}Cl_2NO_2$	A	197							4.82			4.89	1.0
β-Hydroxy-γ-trifluorobutyric	$C_4H_7F_3O_2$	(8)												
2-Hydroxyethyl	$C_4H_9F_2NO_2$	B (8)	59-61											1.1
Vinylacetic	$C_4H_5O_2$	(9)												
2-Hydroxyethyl	$C_4H_{11}NO_2$	A	B.p. 138-142 (1 mm.)	55.79	8.59			55.78	8.59					1.4
Ethylmercureimercaptoacetic	$C_4H_5O_2SHg$	(10)												Toxic
β-Hydroxy-α-butyric	$C_4H_7O_2$	†												
2-Hydroxyethyl	$C_4H_9NO_2$	B	68-71							9.52			9.78	1.0
β-Methoxypropionic	$C_4H_7O_2$	(11)												
2-Hydroxyethyl	$C_4H_{11}NO_2$	B	B.p. 142-145 (1.5 mm.)							9.52			9.75	1.0

	(12)†							
Thiophene-2-carboxylic 2-Hydroxyethyl Allyl	B A "	C ₆ H ₄ O ₈ S C ₇ H ₄ NO ₈ S C ₉ H ₄ NO ₈ S	90-91 65 123-124	8.18 8.38	52.84 52.75	5.76 5.40	7.97 8.29	1.0 1.0 1.0
DL-Valine Allylsulfinylacetic 2-Hydroxyethyl	D	C ₆ H ₄ O ₈ S C ₇ H ₁₀ NO ₈ S C ₉ H ₁₀ NO ₈ S	81.5-82	7.33	43.94	6.90	7.35	1.2
Allylsulfonylacetic 2-Hydroxyethyl	E	C ₆ H ₄ O ₈ S	Oil	6.76			6.72	0.9
3-Ethylmercaptopropionic 2-Hydroxyethyl	C B	C ₇ H ₁₀ O ₈ S C ₇ H ₁₀ NO ₈ S	B.p. 173-175 (0.45 mm.)	7.90			8.07	1.0
Sorbic 2-Hydroxyethyl	† B	C ₆ H ₄ O ₈ C ₇ H ₁₀ NO ₈	B.p. 158-160 (1 mm.)	9.03			9.46	1.1
β-Allyloxypropionic 2-Hydroxyethyl	F (11) B	C ₆ H ₁₀ O ₈ C ₆ H ₁₀ NO ₈	B.p. 142-144 (0.3 mm.)	8.09			8.51	0.8
4-Butylacetic DL-Valine Isocaproic	(13) A †	C ₆ H ₁₀ O ₈ C ₁₁ H ₁₄ NO ₈ C ₆ H ₁₀ O ₈	147-148	6.50			6.40	0.8
DL-Valine γ-Ethoxybutyric 2-Hydroxyethyl	A † B	C ₁₁ H ₁₄ NO ₈ C ₆ H ₁₀ O ₈ C ₆ H ₁₀ NO ₈	100-101 B.p. 138-140 (1 mm.)	6.50 7.99			6.42 8.31	1.0 1.0
p-Chlorobenzoic 2-Hydroxyethyl Allyl	† A "	C ₇ H ₄ ClO ₈ C ₆ H ₁₀ ClNO ₈ C ₉ H ₁₀ ClNO	113-114 73	5.05 5.15	54.26 61.00	5.05 5.10		1.0 1.0 1.0
DL-Valine DL-Alaninyl-DL-valine Benzoic Allyl	" G, † (14) B	C ₁₂ H ₁₄ ClNO ₈ C ₇ H ₄ O ₈ C ₉ H ₁₀ NO C ₁₀ H ₁₂ NO ₈	178-179 204-206 67-69	5.48 8.58 6.27			5.68 8.65 6.75	1.0 1.0 0.9 1.0

TABLE II—Continued

Name of acid and amide	Empirical formula	Method of preparation*	M.p.	Analyses						Stimulation
				Calculated			Found			
				C	H	N	C	H	N	
			°C.	per cent	per cent	per cent	per cent	per cent	per cent	
<i>p</i> -Arsenosphenoxycetic	C ₇ H ₇ O ₄ As	(15)								1.0
Benzylsulfonic	C ₇ H ₉ O ₂ S	†								
DL-Valine	C ₁₃ H ₁₇ NO ₂ S	A	120-123			5.16	66.78	8.06	5.10	1.0
β , β -Dimethyl- γ -pentynoic	C ₇ H ₁₀ O ₂	H	B.p. 75-77 (2 mm.)	66.64	7.99					
2-Hydroxyethyl	C ₉ H ₁₂ NO ₂	B	B.p. 150-155 (0.5 mm.)	63.88	8.94		63.80	8.95		0.8
Cyclopentylacetic	C ₇ H ₁₂ O ₂	†								
2-Hydroxyethyl	C ₉ H ₁₇ NO ₂	B	57-58			8.18			8.15	1.6
Hexahydrobenzoic	C ₇ H ₁₂ O ₂	†								
DL-Valine	C ₁₃ H ₁₇ NO ₂	A	195-197			6.16			6.22	1.0
Benzoylformic	C ₈ H ₈ O ₃	(16)								1.0
Mandelic	C ₈ H ₈ O ₃	†								
2-Hydroxyethyl	C ₁₀ H ₁₃ NO ₂	B	61-64			7.02			7.08	1.0
Phenylselenoacetic	C ₈ H ₉ O ₂ Se	(17)								1.8
2-Hydroxyethyl	C ₁₀ H ₁₃ NO ₂ Se	A	56-58			5.42			5.91	1.2
Phenylsulfonylacetic	C ₈ H ₉ O ₃ S	(18)								1.0
2-Hydroxyethyl	C ₁₀ H ₁₃ NO ₂ S	B	93-94			5.76			5.89	1.1
Phenylarsinicoacetic	C ₈ H ₉ O ₄ As	(19)								Toxic
DL-Valine	C ₁₃ H ₁₈ NO ₄ As	I	188 (Decomposition)			4.08			4.14	1.0
Cyclohexylacetic	C ₈ H ₁₁ O ₂	†								
2-Hydroxyethyl	C ₁₀ H ₁₅ NO ₂	B	66-68			7.57			7.59	1.0
DL-Valine	C ₁₃ H ₁₇ NO ₂	A	178-179			5.80			5.50	1.1

ω -Carbethoxy- <i>n</i> -valeric	$C_9H_{14}O_4$	†	Oil			6.46			6.67	1.0
2-Hydroxyethyl	$C_{10}H_{12}NO_4$	A								0.9
Phenylpropionic	$C_9H_8O_2$	†								
α -Cyanophenylacetic	$C_8H_7NO_2$	(20)								
2-Hydroxyethyl	$C_{11}H_{12}N_2O_2$	B	105-107			13.72			13.63	0.8
<i>m</i> -Trifluoromethylphenylseleno-	$C_8H_7F_3O_2Se$	J	58.5-59.5				38.00	2.68		1.0
acetic										
Cinnamic	$C_9H_8O_2$	†								
2-Hydroxyethyl	$C_{11}H_{12}NO_2$	B	101			7.33			7.39	1.0
DL-Valine	$C_{10}H_{17}NO_2$	A	183-184			5.49			5.59	1.0
Allyl	$C_{12}H_{15}NO$	(14)	90-92			11.19			11.10	0.9
2,4-Dichlorobenzylsulfonyl-	$C_8H_6Cl_2O_2S$	(21)								0.9
acetic										
Phenylmalonic	$C_9H_8O_4$	(22)								
Di-2-hydroxyethyl	$C_{12}H_{18}N_2O_4$	B							10.24	1.2
<i>p</i> -Chlorobenzylsulfonylacetic	$C_8H_7ClO_2S$	(21)	Oil			10.52				0.9
Hydrocinnamic	$C_9H_{10}O_2$	†								
DL-Valine	$C_{10}H_{19}NO_2$	A	141-143			5.63			5.57	1.0
α -Phenylmercaptopropionic	$C_9H_{10}O_2S$	(23)								1.3
β -Phenylmercaptopropionic	$C_9H_{10}O_2S$	(24)								
DL-Valine	$C_{10}H_{19}NO_2S$	A	93-94			4.98			5.14	1.1
O-Methylmandelic	$C_9H_{10}O_3$	(25)								
2-Hydroxyethyl	$C_{11}H_{14}NO_2$	B	84-87			6.67			6.95	1.0
Tropic	$C_9H_{10}O_3$	(26)								
2-Hydroxyethyl	$C_{11}H_{14}NO_2$	B	Oil			6.69			7.20	1.2
Benzylsulfonylacetic	$C_9H_{10}O_2S$	(21)								1.0
N-Phenylsarcosine	$C_9H_{11}NO_2$	(27)								
2-Hydroxyethyl	$C_{11}H_{14}N_2O_2$	B	56-57			13.45			13.53	1.0
<i>p</i> -Chlorocarbonyloxylglycine	$C_8H_6ClNO_2$	(28)	108-109.5			5.75			5.66	1.0

3-Indolepropionic	$C_{11}H_{11}NO_2$	§ (37)							0.9
γ-Benzoylbutyric	$C_{11}H_{12}O_3$	†							1.0
Benzylsuccinic	$C_{11}H_{12}O_4$	(38)							1.0
β-(p-Bromophenyl)-isovaleric	$C_{11}H_{12}BrO_2$	A	109-110		3.93			3.95	0.8
DL-Valine	$C_6H_{12}BrNO_2$	(38)							0.5
β-(p-Chlorophenyl)-isovaleric	$C_{11}H_{11}ClO_2$	(38)							0.9
β-(p-Fluorophenyl)-isovaleric	$C_{11}H_{11}FO_2$	(38)							Toxic
β-(p-Iodophenyl)-isovaleric	$C_{11}H_{11}IO_2$	(38)							
β-(p-Nitrophenyl)-isovaleric	$C_{11}H_{11}NO_4$	(38)							
DL-Valine	$C_6H_{12}N_2O_5$	A	110-115		8.69			8.44	1.3
β-Phenylvaleric	$C_{11}H_{11}O_2$	(39)							
DL-Valine	$C_6H_{12}NO_2$	A	98-100		5.05			5.13	0.8
β-Phenylmercaptovaleic	$C_{11}H_{11}O_2S$	L							
2-Hydroxyethyl	$C_3H_7NO_2S$	B	91-92		5.53			5.77	1.0
β-(p-Hydroxyphenyl)-isovaleric	$C_{11}H_{14}O_3$	(38)							1.0
β-(p-Aminophenyl)-isovaleric	$C_{11}H_{14}NO_2$	(38)							1.0
p-Trimethylsilylphenylseleno- acetic	$C_{11}H_{16}O_2SeSi$		B.p. 170-173 (4 mm.)	45.99	5.61	46.00	5.50		Toxic
Cyclohexylvaleric	$C_{11}H_{20}O_2$	(40)							"
Δ ¹⁰ -Undecylenic	$C_{11}H_{20}O_2$	†							
2-Hydroxyethyl	$C_3H_7NO_2$	B	66-67		6.16			6.14	1.0
γ-(3-Iadole)-butyric	$C_8H_{12}NO_2$	†							
2-Hydroxyethyl	$C_4H_7N_2O_2$	B	Oil		11.38			11.17	1.0
ε-(p-Chlorophenyl)-caproic	$C_{12}H_{15}ClO_2$	M							
2-Hydroxyethyl	$C_4H_9ClNO_2$	B	"		5.19			5.08	1.4
Lauric	$C_{12}H_{24}O_2$	†							
2-Hydroxyethyl	$C_3H_7NO_2$	B (41)	86-87		5.76			5.58	0.9
β-(1-Naphthyl)-propionic	$C_{12}H_{17}O_2$	(42)							
2-Hydroxyethyl	$C_{12}H_{17}NO_2$	B	60-61		5.75			5.85	1.0
6-Benzoyl-3-ketocaproic	$C_{12}H_{14}O_4$	(43)							1.0
γ-Mesitylbutyric	$C_{12}H_{16}O_2$	(44)	82-84	75.69	8.80	75.24	8.60		Toxic

TABLE II—Concluded

Name of acid and amide	Empirical formula	Method of preparation*	M.p. °C.	Analyses						Stimula- tion
				Calculated			Found			
				C per cent	H per cent	N per cent	C per cent	H per cent	N per cent	
Diphenylacetic	C ₁₄ H ₁₂ O ₂	†								
2-Hydroxyethyl	C ₁₆ H ₁₇ NO ₂	B	118-119			5.48			5.43	1.0
Myristic	C ₁₄ H ₂₈ O ₂	†								
2-Hydroxyethyl	C ₁₆ H ₂₉ NO ₂	B (36)	94-95			5.16			5.07	1.0
β,β-Di-(<i>p</i> -chlorophenyl)- propionic	C ₁₈ H ₁₇ Cl ₂ O ₂	N	182-183	61.03	4.09		61.41	4.00		0.4
DL-Valine	C ₂₀ H ₂₁ Cl ₂ NO ₃	A	155-156	60.92	5.37		60.95	5.63		0.9
β,β-Diphenylpropionic	C ₁₈ H ₁₄ O ₂	†								
2-Hydroxyethyl	C ₁₇ H ₁₉ NO ₂	B	94			5.20			5.40	0.9
γ-(4-Methoxy-1-naphthyl)- butyric	C ₁₈ H ₁₆ O ₃	(45)								
2-Hydroxyethyl	C ₁₇ H ₂₁ NO ₂	B	Oil			4.87			4.94	0.6
Dibenzylacetic	C ₁₈ H ₁₆ O ₂	†								
2-Hydroxyethyl	C ₁₈ H ₂₁ NO ₂	B	83-84			4.94			5.27	0.9
Palmitic	C ₁₆ H ₃₂ O ₂	†								
2-Hydroxyethyl	C ₁₈ H ₃₇ NO ₂	B (46)	97.5			4.68			4.66	1.0
β,β-Di-(<i>p</i> -tolyl)-propionic	C ₁₇ H ₁₈ O ₂	(47)								
2-Hydroxyethyl	C ₁₉ H ₂₁ NO ₂	B	85-86			4.71			4.61	0.9
9- <i>p</i> -Iodophenylundecanoic	C ₁₇ H ₂₅ IO ₂	¶								
2-Hydroxyethyl	C ₁₉ H ₃₀ INO ₂	B	Oil			3.25			3.41	1.0
β-Phenylundecanoic	C ₁₇ H ₂₆ O ₂	(48)								
2-Hydroxyethyl	C ₁₉ H ₃₁ NO ₂	B	"			4.58			4.77	1.0
Linoleic	C ₁₈ H ₃₂ O ₂	†								
2-Hydroxyethyl	C ₂₀ H ₃₇ NO ₂	B	B.p. 215-220 (1 mm.)	74.25	11.52	4.33	73.59	11.48	4.63	0.9

β , γ -Di-(<i>p</i> -anisyl)-butyric	$C_{13}H_{20}O_4$	O	167-168	71.97	6.71	3.51	71.67	6.45	3.46	1.0
D,L-Valine	$C_{12}H_{20}NO_3$	A	147-148							
Ricinoleic	$C_{18}H_{34}O_2$	†								
2-Hydroxyethyl	$C_{12}H_{22}NO_3$	B	54-55			4.10			4.12	1.0
9,10-Dihydroxystearic	$C_{18}H_{36}O_4$	(49)								
2-Hydroxyethyl	$C_{10}H_{18}NO_4$	B	150			3.89			4.03	1.4
β -1-Pyrenoylpropionic	$C_{26}H_{34}O_3$	†								Toxic

* The figures in parentheses refer to bibliographic references.

† Commercially available.

‡ Obtained by the catalytic hydrogenation of the nitro compound.

§ Obtained from Parke, Davis and Company.

|| To be published subsequently by R. G. Jones.

¶ "Pantopaque," obtained from the Abbott Laboratories.

absolute alcohol in which had been dissolved 46 gm. (2 gm. atom) of sodium, were added 194 gm. (1.88 mole) of 3,3-dimethyl-3-chloro-1-propyne. The temperature slowly rose to 70° during $\frac{1}{2}$ hour. The mixture was kept at 60–65° for 45 minutes, refluxed for $\frac{1}{2}$ hour, and then allowed to stand overnight at 40°. The mixture was filtered and the filtrate was evaporated under a vacuum to remove most of the alcohol. The residual syrup was treated with dilute ice-cold hydrochloric acid and extracted with ether. The ether solution was washed with water and dilute sodium bicarbonate solution, dried over magnesium sulfate, and the ether evaporated; the remaining liquid was fractionated *in vacuo* through a 15 inch Vigreux column. There were obtained 192 gm. (45 per cent) of 1,1-dimethyl-2-propynylmalonic ester, b.p. 102–104° at 3 mm.

Analysis— $C_{12}H_{18}O_4$. Calculated, C 63.70, H 8.02; found, C 62.92, H, 7.90

A mixture of 190 gm. (0.88 mole) of the above ester, 400 ml. of 6 N sodium hydroxide solution, and 300 ml. of alcohol was warmed and stirred on the steam bath. The resulting solid was dissolved in 1500 ml. of hot water and the solution was partially evaporated under a vacuum on the steam bath to remove the alcohol. The cooled solution was washed with two 300 ml. portions of ether, acidified with sulfuric acid, and extracted with one 300 ml. portion and three 100 ml. portions of ether. The ether solution was dried over magnesium sulfate and the ether was removed, leaving 148 gm. (98 per cent) of crystalline dimethylpropynylmalonic acid, m.p. 105–106° (from ether-petroleum ether).

Analysis— $C_8H_{10}O_4$. Calculated, C 56.47, H 5.92; found, C 55.63, H 5.88

The crude acid (145 gm., 0.85 mole) was heated in a 500 ml. flask in an oil bath at 180–200° for 1 to 2 hours. Decarboxylation took place smoothly. The liquid product was distilled in a vacuum through a 15 inch Vigreux column. It boiled at 75–77° at 2 mm., with a bath temperature of 130°. The yield was 91 gm. (85 per cent).

A solution of 40 gm. (0.317 mole) of β,β -dimethyl- γ -pentynoic acid in 200 ml. of methanol and 10 ml. of concentrated sulfuric acid was allowed to stand at room temperature for 6 days and then worked up in the usual manner to yield 28 gm. (65 per cent) of methyl ester, boiling at 150–153°.

Analysis— $C_8H_{12}O_2$. Calculated, C 68.53, H 8.63; found, C 68.10, H 8.81

Method I. N-Phenylarsinicoacetyl-DL-valine—9.4 gm. of chloroacetylvaline were added with stirring to a solution of 10 gm. of phenyldichloroarsine in 30 ml. of 10 N sodium hydroxide solution according to the procedure of Quick and Adams (19). After 3 hours of stirring the solution was diluted with an equal volume of water, the precipitate was removed by filtration, and hydrochloric acid was added until the filtrate was neutral to phenol-

phthalein. After filtration, the solution was acidified to methyl orange. The precipitated oil crystallized when cooled. Recrystallization from water yielded 8.5 gm. of product, melting with evolution of a gas at 188°.

Method J. *m*-Trifluoromethylphenylselenoacetic Acid—The Grignard reagent was prepared from 0.15 mole of *m*-trifluoromethylbromobenzene (54) with an excess of magnesium in dry ether. With stirring 0.15 gm. atom of selenium powder was added in portions during 10 minutes (17). The mixture was stirred for 15 minutes and then hydrolyzed by the dropwise addition of 25 ml. of water, followed by 100 ml. of 2 N hydrochloric acid. The ether layer was separated, washed with water, and extracted with 65 ml. of 3 N sodium hydroxide solution. The aqueous extract was added to a solution of 0.15 mole of sodium chloroacetate in 100 ml. of water. After a few minutes the mixture was acidified with hydrochloric acid and extracted with ether. The ether solution was washed with water and extracted with sodium bicarbonate solution, and the aqueous extract was acidified. The precipitated oil was collected in ether, dried, and distilled. The acid boiled at 140–142° at 7 mm., and crystallized after standing; m.p. 58.5–59.5°.

Method K. γ -Phenylmercaptobutyric Acid—A mixture of 28 gm. of sodium methoxide, 300 ml. of absolute ethanol, 56 gm. of thiophenol, and 52 gm. of γ -chlorobutyronitrile was boiled under a reflux and stirred overnight. The alcohol was then removed by distillation and water and ether were added to the residue. The organic layer was washed with water and dilute alkali. Distillation *in vacuo* gave a fraction boiling at 135–137° at 0.1 mm. (72 gm.) which was not analytically pure. However, hydrolysis of 31 gm. of this semipure nitrile with 16 gm. of potassium hydroxide, 20 ml. of water, and 100 ml. of ethanol gave γ -phenylmercaptobutyric acid on dilution and acidification; m.p. 58–60°.

Method L. Ethyl δ -Phenylmercaptovalerate—34 gm. of thiophenol and 58 gm. of ethyl δ -bromovalerate were added to a solution of sodium ethoxide prepared from 6.9 gm. of sodium in 200 ml. of absolute alcohol. Stirring and refluxing were maintained overnight, whereupon the alcohol was distilled and the residue was treated with water. The organic layer was separated with ether, washed with dilute sodium hydroxide solution, then with water, and dried over magnesium sulfate. The ester obtained on distillation boiled at 121–124° at 0.2 mm.

Analysis— $C_{11}H_{14}O_2S$. Calculated, C 65.51, H 7.61; found, C 65.30, H 7.48

Method M. Methyl δ -*p*-Chlorophenyl-*n*-caproate—Ethyl hydrogen adipate (87 gm.) was converted to the acid chloride with 80 ml. of thionyl chloride. After removal of excess thionyl chloride, the crude product was treated with 100 gm. of chlorobenzene, 135 gm. of anhydrous aluminum chloride, and 350 ml. of carbon disulfide in the usual Friedel-Crafts manner.

The yield of ethyl δ -*p*-chlorobenzoylvalerate was 66.4 gm. After several recrystallizations from ether-petroleum ether, it melted at 59–60°.

Analysis— $C_{14}H_{17}ClO_3$. Calculated, C 62.56, H 6.37; found, C 61.82, H 6.30

Saponification of 53 gm. of ester with potassium hydroxide gave 36 gm. of δ -*p*-chlorobenzoylvaleric acid which could be recrystallized from dilute ethanol or ethanol-ether-petroleum ether, m.p. 134–136°.

Analysis— $C_{13}H_{15}ClO_3$. Calculated, C 59.88, H 5.44; found, C 59.66, H 5.36

36 gm. of δ -*p*-chlorobenzoylvaleric acid was heated under a reflux for 22 hours with 60 gm. of amalgamated zinc, 38 ml. of water, 90 ml. of concentrated hydrochloric acid, and 75 ml. of toluene (44). After cooling, ether was added and the organic layer was separated. The ethereal solution was extracted with dilute sodium hydroxide solution and then the aqueous extract was acidified. The crude acid was treated with methanol and sulfuric acid, yielding the ester; b.p. 122–125° at 0.4 mm.

Analysis— $C_{13}H_{17}ClO_2$. Calculated, C 64.85, H 7.12; found, C 64.75, H 7.12

Method N. β,β -Di-*p*-chlorophenylpropionic Acid—Di-*p*-chlorobenzophenone (0.3 mole) was converted to ethyl β -hydroxy- β,β -di-*p*-chlorophenylpropionate, m.p. 96–97°, in 90 per cent yield by the Reformatsky method (55).

Analysis— $C_{17}H_{18}Cl_2O_3$. Calculated, C 60.19, H 4.76; found, C 60.71, H 4.71

The hydroxy ester (30 gm.) was dehydrated by warming in 100 ml. of benzene with 25 gm. of phosphorus pentoxide for 1 hour and the resulting unsaturated ester was saponified with sodium hydroxide solution to yield β,β -di-*p*-chlorophenylacrylic acid, m.p. 173–174°.

Analysis— $C_{16}H_{16}Cl_2O_2$. Calculated, C 61.46, H 3.43; found, C 61.23, H 3.46

The acid (29.3 gm., 0.10 mole) was hydrogenated in alcohol over 5 per cent palladium on charcoal at 4 atmospheres until exactly 0.10 mole of hydrogen was absorbed. The yield of β,β -di-*p*-chlorophenylpropionic acid, after crystallization from 150 ml. of benzene, was 21 gm. (71 per cent); m.p. 182–183°.

Method O. β,γ -Di-*p*-Anisylbutyric Acid—Desoxyanisoin (77 gm., 0.3 mole) and 50 gm. (0.3 mole) of ethyl bromoacetate in 150 ml. of dry benzene were heated to boiling and treated with 30 gm. of zinc dust added in small portions. After refluxing for 1 hour the mixture was cooled, shaken with dilute sulfuric acid, and the benzene layer together with the benzene washings dried over magnesium sulfate. The benzene was removed and the residual product distilled *in vacuo*, whereupon dehydration took place and ethyl β,γ -di-*p*-anisylbutenoate was obtained as a viscous liquid, b.p. 221° at 2 mm. The yield was 88 gm. (90 per cent).

Analysis— $C_{20}H_{24}O_4$. Calculated, C 73.59, H 6.79; found, C 73.83, H 6.68

This ester was hydrogenated in alcohol over 5 per cent palladium on charcoal at 4 atmospheres and the reduced mixture was saponified with sodium hydroxide to furnish β,γ -di-*p*-anisylbutyric acid in 90 per cent yield; m.p. 167–168°.

The authors express their gratitude to Dr. G. H. A. Clowes and Dr. E. C. Kleiderer for their interest in this work.⁸ The microanalyses were performed by W. L. Brown and H. L. Hunter.

SUMMARY

Methods are described for the evaluation of compounds as precursors for new penicillins. These include stimulation tests, differential assays, distribution studies with the "Craig machine" or with silica-buffer partition columns, and isolation work.

Data obtained by these methods are presented for a considerable group of compounds including aryl carboxylic acids, α -substituted phenylacetic acids, aliphatic acids, aryl aliphatic (other than acetic) acids, a miscellaneous unclassified group of acids, and certain derivatives of these acids.

The preparation and some properties of new compounds belonging to the above series are presented.

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⁸ Valuable technical assistance has been given by Lynnette Garrison, Charlotte Harris, Charleen W. McClain, John O'Brien, and F. R. Van Abeele.

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BIOSYNTHESIS OF PENICILLINS

IV. NEW CRYSTALLINE BIOSYNTHETIC PENICILLINS

By OTTO K. BEHRENS, JOSEPH CORSE,* JOHN P. EDWARDS, LYNETTE GARRISON, REUBEN G. JONES, QUENTIN F. SOPER, F. R. VAN ABEELE, AND CALVERT W. WHITEHEAD

(From the Lilly Research Laboratories, Indianapolis)

(Received for publication, February 28, 1948)

Paper III (1) in this series describes several tests which were found to be useful in determining whether a given compound is utilized by the mold in penicillin formation. To provide proof of the incorporation of various acyl groups into the penicillin molecule and to be able to determine the chemical characteristics and biological properties of the various penicillins, isolation and crystallization were required.

Table V in chapter 19 of the penicillin monograph (2) lists some properties of eleven new penicillins. The experimental procedure followed in the preparation of these penicillins is described here. Eighteen additional new penicillins have been crystallized and characterized (*cf.* Table I). One other penicillin, γ -chloroallylmercaptomethylpenicillin has been partially purified but attempts to obtain a crystalline product have been unsuccessful.

Each of the penicillins listed except Penicillins 4, 13, 15, 17, and 18 gave satisfactory analytical values. Additional identification was provided in many cases by comparison of the ultraviolet absorption curves of the penicillin and its precursor. It will be noted that *p*-bromobenzylpenicillin was isolated following the use of N-(γ -*p*-bromophenylbutyryl)-DL-valine as precursor. It is apparent that γ -substituted butyric acid derivatives are effective in a manner similar to the corresponding substituted acetic acid derivatives and must undergo oxidative degradation with the loss of 2 carbon atoms.

Experimental details of the preparation of the precursors used in this work will appear in other papers of this series. Extensive biological tests have been undertaken to compare the properties of natural and biosynthetic penicillins. These studies will be reported elsewhere.

EXPERIMENTAL

p-Methoxybenzylpenicillin¹—N-(2-Hydroxyethyl)-*p*-methoxyphenylacetamide (170 mg.) (3) was added to a broth containing 25 gm. of lactose,

* Present address, University of California, Los Angeles, California.

¹ A number of these penicillins have been described in reports to the Office of Scientific Research and Development.

20 gm. of corn steep solids, 2 gm. of calcium carbonate, and 0.044 gm. of zinc sulfate heptahydrate per liter. 200 ml. of broth in each 1 liter Erlenmeyer flask were inoculated with 10 ml. of a 2 day vegetative inoculum of

TABLE I
New Crystalline Biosynthetic Penicillins

Penicillin formed	Precursor used	Activity	Differential assay*
		units per mg.	
1. Cyclopentylmethyl-	N-(2-Hydroxyethyl)-cyclopentylacetamide	2080	0.72
2. <i>p</i> -Methylbenzyl-	N-(2-Hydroxyethyl)- <i>p</i> -tolylacetamide	2280	0.73
3. <i>p</i> -Allyloxybenzyl-	N-(2-Hydroxyethyl)- <i>p</i> -allyloxyphenylacetamide	1440	0.87
omethyl-	Methylmercaptoacetic acid	550 (?)	1.50
nethyl-	Ethylmercaptoacetic "	1310	0.93
ptomethyl-	<i>n</i> -Propylmercaptoacetic "	2550	0.55
ptomethyl-	Isopropylmercaptoacetic "	1900	0.72
8. Allylmercaptomethyl-	N-(2-Hydroxyethyl)-allylmercaptoacetamide	1630	0.76
mercapto-	β -Bromoallylmercaptoacetic acid	2030	0.71
methyl-			
10. <i>n</i> -Butylmercaptomethyl-	<i>n</i> -Butylmercaptoacetic acid	3400	0.53
11. Isoamylmercaptomethyl-	Isoamylmercaptoacetic "	2900	0.54
12. <i>m</i> -Trifluoromethylphenylmercaptomethyl-	<i>m</i> -Trifluoromethylphenylmercaptoacetic acid	1900	0.86
13. γ -Phenylpropylmercaptomethyl-	γ -Phenylpropylmercaptoacetic acid	1600 (?)	0.54
14. β -Phenoxyethylmercaptomethyl-	β -Phenoxyethylmercaptoacetic acid	1190	0.84
15. β -Naphthylmercaptomethyl-	N-(2-Hydroxyethyl)- β -naphthylmercaptoacetamide	2160 (?)	0.91
16. Phenylselenomethyl-	Phenylselenoacetic acid	2660	0.74
17. <i>p</i> -Methoxyphenoxy-methyl-	N-(2-Hydroxyethyl)- <i>p</i> -methoxyphenoxyacetamide	1120	0.92
18. 3-Thiophenemercaptomethyl-	3-Thiophenemercaptoacetic acid	2000	0.76

* The differential assay represents the ratio of activity of *Bacillus subtilis* to *Staphylococcus aureus* compared with that for pure benzylpenicillin, which is defined as 1.00. We acknowledge with many thanks the numerous assays performed by Dr. J. M. McGuire.

Penicillium notatum, strain NRRL 1976, grown on a similar medium without the precursor. The flasks were shaken at 25° for 4 days and harvested. 5.1 liters of cold filtered broth, assaying 83 units per ml., were

extracted at pH 2.2 with 0.6 volume of amyl acetate. The further purification was carried out in a similar manner to that described for benzylpenicillin (4). The principal band on the ether column was in a position similar to that occupied by benzylpenicillin on other comparable columns and represented 79 per cent of the penicillin recovered from the column. The principal band on the chloroform column represented 92 per cent of the recovered units. 60 mg. of sodium salt were obtained on drying from the frozen state. On treatment with 1 ml. of acetone partial solution followed by reprecipitation occurred. The material was washed with 5 ml. of absolute acetone and was crystallized in the form of needles from 1 ml. of 90 per cent aqueous acetone by addition of 4 ml. of acetone. The sodium *p*-methoxybenzylpenicillin thus obtained assayed 1510 units per mg. and gave a differential assay value of 0.82. $[\alpha]_D^{30} = +278^\circ$ (0.3 per cent in water). The ultraviolet absorption curve was in good agreement with that observed for the precursor.²

Analysis— $C_{17}H_{19}N_2O_6SNa$. Calculated. C 52.86, H 4.92, N 7.25, OCH_3 8.03
Found. " 52.51, " 4.99, " 7.20, " 7.95

2-Thiophenemethylpenicillin—The precursor used to obtain this penicillin was *N*-(2'-hydroxyethyl)-2-thiopheneacetamide (150 mg. per liter) (5). The procedure followed in the culture and purification was similar to that described for *p*-methoxybenzylpenicillin. 91 per cent of the penicillin recovered from the ether-silica column was in a single band which occupied a position similar to that in which benzylpenicillin is found in comparable columns. The principal band on the chloroform column contained 95 per cent of the total activity. The sodium salt, dried from the frozen state, weighed 213 mg. and assayed 1400 units per mg. The dry material was washed repeatedly with small portions of acetone. Crystallization in clusters of plate-like crystals occurred on solution in 2 ml. of 90 per cent acetone, followed by addition of 2 ml. of dry acetone. The sodium 2-thiophenemethylpenicillin thus obtained assayed 1685 units per mg. and gave a differential assay value of 1.13. $[\alpha]_D^{30} = +265^\circ$ (0.3 per cent in water). The ultraviolet absorption curve was in good agreement with that observed for the precursor with a characteristic absorption at 234 $m\mu$.²

Analysis— $C_{14}H_{15}N_2O_4S_2Na$. Calculated. C 46.40, H 4.17, N 7.73, S 17.69
Found. " 46.46, " 3.81, " 7.40, " 17.18

p-Chlorobenzylpenicillin—*p*-Chlorophenylacetyl-DL-valine (220 mg. per liter) (3) was used as the precursor for this penicillin. The procedure previously outlined for the preparation of *p*-methoxybenzylpenicillin was

² See the figure, chapter 19 (2).

followed. The principal band on the ether-silica column occupied a position approximately similar to that occupied by *n*-heptylpenicillin in control columns. 92 per cent of the recovered units were found in this band. 97 per cent of the units recovered from the chloroform column were present in fractions that gave some indication of the presence of two peaks. The material was rechromatographed on a second chloroform-silica column, and, in spite of more extensive development of this column, showed no evidence of separation into two bands. A light yellow solid was obtained on drying the sodium salt from the frozen state. The solid material was washed with several portions of acetone and was crystallized (large clumps of needles) from 2 ml. of 90 per cent acetone by addition of 4 ml. of acetone. After one recrystallization the sodium *p*-chlorobenzylpenicillin assayed 2450 units per mg. and gave a differential assay value of 0.73. $[\alpha]_D^{80} = +260^\circ$ (0.3 per cent in water). The ultraviolet absorption curve was in good agreement with that observed for the precursor.²

Analysis— $C_{16}H_{18}ClN_2O_4SNa$. Calculated. C 49.17, H 4.12, N 7.17, Cl 9.07
Found. " 49.08, " 3.84, " 7.30, " 8.85

p-Nitrobenzylpenicillin—Both N-(*p*-nitrophenylacetyl)-DL-valine (230 mg. per liter) (3) and N-(*p*-nitrophenylacetyl)-DL-isoleucine (3) have been used as precursors. With the former compound the following results were obtained. The desired band on the ether-silica column contained 38 per cent of the recovered units and was located just above the band of 2-pentenylpenicillin. No further separation into bands occurred on the chloroform column. The sodium salt was dried from the frozen state. The material was soluble in acetone, allowing separation of a small amount of inorganic material. After several hours, crystallization took place in clusters of very fine needles. Due to insufficient material, recrystallization was not attempted. The ultraviolet absorption spectrum was in good agreement with that found for the precursor with a characteristic peak at 276 $m\mu$.² The material assayed about 1700 units per mg. and gave a differential assay value of 0.86.

p-Fluorobenzylpenicillin—The precursor used to obtain this penicillin was N-(2-hydroxyethyl)-*p*-fluorophenylacetamide (160 mg. per liter) (3). 80 per cent of the penicillin recovered from the ether-silica (pH 6.2) chromatographic column was in a single band. When this material was subjected to further purification on a similar chloroform column, 98 per cent of the recovered activity was found in a single band. The sodium salt was prepared by use of 0.1 N sodium hydroxide solution, and was dried from the frozen state. Solution and precipitation occurred on treatment with dry acetone. The solid material was purified by two crystallizations from 90 per cent acetone by addition of 4 parts of absolute acetone. The

sodium *p*-fluorobenzylpenicillin thus obtained assayed 1650 units per mg. and gave a differential assay value of 0.89. The ultraviolet absorption curve of the penicillin and its precursor are recorded in the penicillin monograph.²

Analysis— $C_{18}H_{18}FN_2O_4SNa$. Calculated. C 51.33, H 4.31, N 7.49, S 8.56
Found. " 51.27, " 4.15, " 7.49, " 8.43

m-Fluorobenzylpenicillin—N-(2-Hydroxyethyl)-*m*-fluorophenylacetamide (160 mg. per liter) (3) was used as the precursor. The principal band on the ether-silica buffer (pH 6.2) chromatographic column contained 93 per cent of the recovered units. On the chloroform column, 98 per cent of the penicillin separated in a single band. A light yellow solid was obtained when the sodium salt was dried from the frozen state. The material was treated with absolute acetone, and was twice crystallized from 0.4 ml. of 90 per cent acetone by addition of 1 ml. of dry acetone. The sodium *m*-fluorobenzylpenicillin thus obtained assayed about 2220 units per mg. and gave a differential assay value of 0.76. The penicillin monograph² contains the ultraviolet absorption curves for this penicillin and its precursor.

Analysis— $C_{18}H_{18}FN_2O_4SNa$. Calculated. C 51.33, H 4.31, N 7.49, S 8.56
Found. " 51.47, " 4.19, " 7.61, " 8.21

o-Fluorobenzylpenicillin—The precursor used was N-(2-hydroxyethyl)-*o*-fluorophenylacetamide (160 mg. per liter) (3). The activity recovered from the ether-silica column separated in a single band. In the subsequent chloroform column, 95 per cent of the recovered activity was in a single band. The lyophilized sodium salt was crystallized by treatment with absolute acetone. After washing with absolute acetone, the sodium *o*-fluorobenzylpenicillin was purified by recrystallizing twice from 90 per cent acetone by addition of 3 volumes of absolute acetone. The purified material assayed 1340 units per mg. and gave a differential assay value of 1.08. The ultraviolet absorption spectrum was in good agreement with that found for the precursor.²

Analysis— $C_{18}H_{18}FN_2O_4SNa$. Calculated. C 51.33, H 4.31, N 7.49, S 8.56
Found. " 51.39, " 4.40, " 7.65, " 8.57

p-Bromobenzylpenicillin—The precursor used to obtain this penicillin was N-(*p*-bromophenylbutyryl)-DL-valine (270 mg. per liter) (1). Assays on the various fractions and percolates of the ether-silica column and the chloroform-silica column showed the presence of only one band. The penicillin recovered from the chloroform column was extracted as the free acid with ether and converted to the sodium salt. The aqueous

solution was dried from the frozen state, and the yellow, hygroscopic powder thus obtained was treated with absolute acetone. Crystallization occurred. The material was purified by two crystallizations from 90 per cent acetone by addition of $2\frac{1}{2}$ parts of absolute acetone. The sodium *p*-bromobenzylpenicillin thus obtained assayed 2270 units per mg. and gave a differential assay value of 0.65. The ultraviolet absorption curves of this penicillin and of the precursor, N-(2-hydroxyethyl)-*p*-bromophenylacetamide, are given in the penicillin monograph.²

Analysis— $C_{16}H_{16}BrN_2O_4SNa$. Calculated. C 44.14, H 3.71, N 6.44
 Found. " 44.36, " 3.93, " 6.55
 $C_{13}H_{20}BrN_2O_4SNa$. Calculated, C 46.66, H 4.35, N 6.08

p-Iodobenzylpenicillin—The precursor used was N-(2-hydroxyethyl)-*p*-iodophenylacetamide (240 mg. per liter) (3). 93 per cent of the activity recovered from the ether column appeared in a single band. No further separation into bands occurred on the chloroform-silica column. The lyophilization and crystallization were carried out in a manner similar to that previously described for benzylpenicillin. The supply of this penicillin was inadequate for proper purification.

Analysis— $C_{16}H_{16}IN_2O_4SNa$. Calculated. N 5.81, I 26.32, S 6.65
 Found. " 6.58, " 22.12

The ultraviolet absorption spectrum agreed well with that found for the precursor with a characteristic peak at 233 $m\mu$.² The preparation was repeated on a larger scale with the same precursor (320 mg. per liter) with strain Q-176. The purification and crystallization were effected in a similar manner. After repeated recrystallizations the *p*-iodobenzylpenicillin assayed 2425 units per mg.

Analysis—Found, N 5.79, I 25.54, S 6.79

Phenoxymethylpenicillin—N-(2-Hydroxyethyl)-phenoxyacetamide (150 mg. per liter) (6) was used as the precursor for this penicillin. The principal band on the ether-silica column (buffer pH 6.2) was at the bottom of the column and in the percolates. It contained 100 per cent of the total units recovered. The chloroform-silica column (pH 6.4) also showed no evidence of more than one band. The penicillin again appeared predominantly in the percolates. Lyophilization of the sodium salt gave a dark yellow powder which was crystallized in absolute acetone. Most of the color was removed by washing the crystalline material with several portions of acetone. Further purification was effected by two crystallizations from 85 per cent acetone by addition of 3 parts of acetone. The sodium phenoxymethylpenicillin assayed about 1670 units per mg. and

gave a differential assay value of 0.87. The ultraviolet absorption spectrum of this penicillin and its precursor are recorded in the monograph.²

Analysis— $C_{16}H_{17}N_2O_4SNa$. Calculated. C 51.60, H 4.60, N 7.52, S 8.61
Found. " 51.17, " 4.49, " 7.59, " 8.50

p-Tolylmercaptomethylpenicillin—The precursor used was N-(*p*-tolylmercaptoacetyl)-DL-valine (230 mg. per liter) (6). Two ether-silica columns with a commercial silica were used in the purification. The distribution of penicillin on these columns and on the subsequent chloroform-silica column was similar to that for *n*-heptylpenicillin and indicated that a high percentage of the penicillin present was the desired *p*-tolylmercaptomethylpenicillin. The lyophilized sodium salt was dissolved in absolute acetone. Undissolved material was removed by filtration and the acetone was evaporated *in vacuo*. After standing *in vacuo* overnight some crystallization was apparent. On being scratched with a glass rod, the material set to a crystalline mass. Most of the color was removed by washing several times with absolute acetone. An attempt was made to effect further purification through recrystallization from 90 per cent acetone by addition of absolute acetone. The material recovered in this manner was amorphous. Characterization was, therefore, carried out with the unrecrystallized product. The ultraviolet absorption spectrum agrees well with that found for the precursor, with a characteristic peak at 250 $m\mu$.² The nitrogen and sulfur analyses indicated lack of purity, owing probably to the presence of inorganic materials. The atomic ratio of sulfur to nitrogen confirmed the presence of a 2nd sulfur atom and indicated that the desired *p*-tolylmercaptomethylpenicillin had been produced.

Analysis—Sodium benzylpenicillin, $C_{16}H_{17}N_2O_4SNa$

Calculated, N 7.83, S 8.98, S:N, 0.5

Sodium *p*-tolylmercaptomethylpenicillin, $C_{17}H_{18}N_2O_4S_2Na$

Calculated. N 6.96, S 15.93, S:N 1.0

Found. " 5.77, " 13.04, " 0.99

The preparation was repeated on a larger scale with *p*-tolylmercaptoacetic acid as precursor (218 mg. per liter) with strain Q-176. The purification and crystallization were effected in a similar manner. The material was recrystallized twice by solution in 2 parts of 90 per cent aqueous acetone, followed by addition of 10 parts of acetone. It was recrystallized once more by dissolving in 2 parts of water, followed by addition of 15 parts of *n*-butanol and evaporation *in vacuo* to 0.3 volume. The tolylmercaptomethylpenicillin assayed 2050 units per mg. and gave a differential assay value of 0.72.

Analysis—Found, N 6.57, S 14.37, S:N, 0.96

Cyclopentylmethylpenicillin—31 gm. of N-(2-hydroxyethyl)-cyclopentyl-acetamide (1) were added to a medium containing 6000 gm. of corn steep liquor, 4500 gm. of lactose, 750 gm. of glucose, 1500 gm. of calcium carbonate, 225 gm. of disodium phosphate, 150 gm. of urea, 37.5 gm. of magnesium sulfate heptahydrate, 0.84 gm. of zinc sulfate, and 40 gallons of distilled water. After sterilization, the medium was inoculated with approximately 1.3 gallons of vegetative growth of *Penicillium chrysogenum* Q-176 and was incubated at 24° with aeration and stirring. After 50 hours, the broth assaying 283 units per ml. was filtered, cooled to 2°, acidified to pH 2.08, and extracted with 0.6 volume of amyl acetate. The 29 gallons of amyl acetate were treated with three successive 0.75 gallon portions of 0.3 M phosphate buffer, pH 8.5. The recovered buffer was cooled to 2°, adjusted to pH 2.1 with 85 per cent phosphoric acid, and extracted successively with 2840 ml., 1420 ml., and 945 ml. of ether. The ethereal solution (4250 ml.) contained 39,000,000 units.

The penicillin was further purified by use of a 4 inch chromatographic column (4) containing 2500 gm. of silica and 1860 ml. of 1.5 M phosphate buffer, pH 6.3. Successive 2 liter portions of ether containing 1, 2, 2.5, and 3 per cent methanol were used to develop the column. Filtrates were collected in 1 liter portions. 33,500,000 units were recovered in a single band near the top of the column. These fractions were combined, extracted with successive 2840, 1420, and 945 ml. portions of cold chloroform at pH 2.1, and were further purified by use of a chloroform-phosphate buffer (pH 6.0) column. 4 liters of chloroform containing 1.5 per cent methanol were used to develop the column. The rather broad band was combined, and the penicillin was extracted from the buffer solution with ether at pH 2.1.

The sodium salt was prepared with 0.1 N sodium hydroxide solution and was dried from the frozen state, yielding 27,000,000 units of amorphous yellow powder. The cyclopentylmethylpenicillin precipitated on treatment with acetone. It was recrystallized by solution in 55 ml. of 87.5 per cent aqueous acetone, followed by addition of 200 ml. of acetone, yielding 10.6 gm., 1880 units per mg., differential assay 0.72.

Analysis— $C_{15}H_{21}N_2O_4SNa$. Calculated. C 51.71, H 6.08, N 8.04
Found. " 50.64, " 6.02, " 7.69

A small sample was recrystallized two additional times in similar fashion and then assayed 2080 units per mg. and gave a differential assay value of 0.72.

Analysis—Found, C 51.69, H 6.07, N 8.21

p-Methylbenzylpenicillin—N-(2-Hydroxyethyl)-*p*-tolylacetamide (155 mg. per liter) (3) was used as the precursor with strain NRRL 1976. The

procedure previously outlined for preparation of *p*-methoxybenzylpenicillin was followed. The penicillin was found in a rather sharp band on the chromatographic column, indicating predominantly the presence of a single penicillin. The purified penicillin was dried from the frozen state, and the amorphous yellow powder was crystallized by treatment with absolute acetone. Further purification was effected by two recrystallizations from 88 per cent acetone by addition of 2 parts of absolute acetone. The *p*-methylbenzylpenicillin thus obtained assayed about 2280 units per mg. and gave a differential assay value of 0.73. The ultraviolet absorption curve demonstrated the presence of the desired penicillin.³

Analysis— $C_{17}H_{19}N_2O_4SNa$. Calculated. C 55.12, H 5.17, N 7.56, S 8.66
Found. " 55.12, " 5.43, " 7.49, " 8.56

p-Allyloxybenzylpenicillin—N-(2-Hydroxyethyl)-*p*-allyloxyphenylacetamide (190 mg. per liter) (3) was used as the precursor with strain NRRL 1976. The purification procedure was similar to that previously described for *p*-methoxybenzylpenicillin. The dried sodium salt was crystallized by treatment with absolute acetone. The crystalline material was washed several times with acetone and was further purified by two recrystallizations effected by dissolving in 90 per cent aqueous acetone and adding 4 volumes of absolute acetone. The *p*-allyloxybenzylpenicillin assayed 1440 units per mg. and gave a differential assay value of 0.87. The ultraviolet absorption curve was in good agreement with that observed for the precursor.³

Analysis— $C_{19}H_{21}N_2O_4SNa$. Calculated. C 55.33, H 5.14, N 6.79
Found. " 55.78, " 5.20, " 6.86

Methylmercaptomethylpenicillin—Methylmercaptoacetic acid (7) (127 mg. per liter) served as the precursor in a fermentation similar to that described for cyclopentylmethylpenicillin. A buffer of pH 6.0 was used with the ether chromatographic column. Fractions near the top of the column with differential assay values of about 1.5 were combined for further purification with a chloroform chromatographic column, buffer at pH 5.8. The fractions with the desired differential assay were combined and converted to sodium salt as described before. The dried material was crystallized by treatment with acetone. Recrystallization was attempted by dissolving in 3 parts of 88 per cent acetone and adding absolute acetone. As the precipitate was somewhat oily, the entire mixture was evaporated to dryness *in vacuo*. The residue was dissolved in 2 parts of water, 10 parts

³ The ultraviolet absorption data were obtained by Dr. W. W. Davis, T. V. Parke, and R. A. Kern. Additional data will be presented in a subsequent publication by these workers.

of *n*-butanol were added, and the water was removed by evaporation *in vacuo*. The methylmercaptomethylpenicillin assayed 550 units per mg. and gave a differential assay value of 1.5.

Analysis— $C_{11}H_{13}N_2O_4S_2Na$. Calculated. S 19.65, N 8.58
Found. " 18.24, " 8.22

Ethylmercaptomethylpenicillin—The fermentation was carried out with ethylmercaptoacetic acid⁴ (144 mg. per liter) in a manner similar to that described for cyclopentylmethylpenicillin. The ether-buffer (pH 6.2) chromatographic column contained only one band of any significance. The chloroform-buffer (pH 6.0) column also contained a single band. The recovered penicillin was dried, yielding 18 gm. of amorphous material. Following treatment with acetone and recrystallization from 87.5 per cent acetone by addition of absolute acetone, the ethylmercaptomethylpenicillin assayed 1310 units per mg. and gave a differential assay value of 0.93.

Analysis— $C_{12}H_{17}N_2O_4S_2Na$. Calculated. S 18.84, N 8.23
Found. " 18.42, " 8.23

n-Propylmercaptomethylpenicillin—*n*-Propylmercaptoacetic acid (7) (24 gm.) was used as the precursor with *Penicillium chrysogenum* Q-176 with the following synthetic medium: 4500 gm. of lactose, 750 gm. of glucose, 750 gm. of ammonium nitrate, 600 gm. of acetic acid, 300 gm. of monopotassium phosphate, 75 gm. of magnesium sulfate heptahydrate, 30 gm. of ferrous sulfate heptahydrate, 0.75 gm. of cupric sulfate pentahydrate, 1.7 gm. of zinc sulfate, 40 gallons of water, and potassium hydroxide to adjust the pH to 5.95. The fermentation and purification were performed in a manner similar to that described for cyclopentylmethylpenicillin except that the ether chromatographic column was run with buffer of pH 6.4 and the chloroform column was omitted. The large band on the center of the ether column was extracted at pH 2.2 with three 2500 ml. portions of cold chloroform, and the sodium salt was prepared by use of 0.1 *N* sodium hydroxide solution. The dried penicillin crystallized readily on addition of acetone. After one recrystallization from 85 per cent acetone by addition of 4 parts of acetone, the material assayed 2300 units per mg. Analysis showed the presence of 16.41 per cent sulfur. After several recrystallizations, the *n*-propylmercaptomethylpenicillin assayed 2550 units per mg. and gave a differential assay value of 0.55.

Analysis— $C_{13}H_{19}N_2O_4S_2Na$. Calculated. S 18.09, N 7.90
Found. " 17.84, " 7.78

Isopropylmercaptomethylpenicillin—Isopropylmercaptoacetic acid (8) (158 mg. per liter) served as the precursor in a fermentation similar to that

⁴ Eastman Kodak Company.

described for cyclopentylmethylpenicillin. The ether-buffer (pH 6.2) column contained a very large band near the top of the column, representing at least 80 per cent of the recovered units. No further separation occurred on the chloroform-buffer (pH 6.2) column. The dried sodium salt was crystallized by treatment with absolute acetone. Recrystallization was effected by dissolving in 90 per cent acetone and adding absolute acetone. The isopropylmercaptomethylpenicillin assayed 1900 units per mg. and gave a differential assay of 0.72.

Analysis— $C_{13}H_{19}N_2O_4S_2Na$. Calculated. S 18.09, N 7.90
Found. " 17.53, " 7.75

Allylmercaptomethylpenicillin—N-(2-Hydroxyethyl)-allylmercaptoacetamide (140 mg. per liter) (6) served as the precursor with strain Q-176. The procedure previously outlined for preparation of *p*-methoxybenzylpenicillin was followed except that the medium contained 3 per cent of corn steep solids, 2.5 per cent of lactose, and 0.5 per cent of calcium carbonate. Both the ether and chloroform columns contained a single, rather sharp band. Crystallization of the dried penicillin occurred on treating the material with absolute acetone. The crystalline material was collected by centrifugation and was washed several times with absolute acetone. After two recrystallizations from 95 per cent aqueous acetone by addition of acetone, the allylmercaptomethylpenicillin assayed 1630 units per mg. and gave a differential assay of 0.76.

Analysis— $C_{13}H_{17}N_2O_4S_2Na$. Calculated. N 7.95, S 18.20
Found. " 8.04, " 18.24

Allylmercaptomethylpenicillin has also been isolated following the use of allylmercaptoacetic acid as the precursor.

β -Bromoallylmercaptomethylpenicillin— β -Bromoallylmercaptoacetic acid (250 mg. per liter) (6) was used as the precursor in a fermentation similar to that described for cyclopentylmethylpenicillin. The ether-buffer (pH 6.3) column contained a rather broad band of activity which gave differential assay values of about 0.8. The band on the chloroform-buffer (pH 6.1) column was of similar character. The entire band was collected and dried as previously described. Upon addition of acetone to the dried material, crystallization occurred slowly. After one recrystallization from 96 per cent aqueous acetone by addition of several parts of absolute acetone the material assayed 1840 units per mg. and gave a differential assay of 0.77.

Analysis— $C_{13}H_{15}BrN_2O_4S_2Na$. Calculated. Br 18.53, S 14.87, C 36.20, H 3.74
Found. " 16.78, " 14.31

A small sample was recrystallized by solution in 90 per cent aqueous *tert*-butanol, followed by addition of absolute *tert*-butanol. After an ad-

ditional similar recrystallization, the material was recrystallized twice from 96 per cent aqueous acetone by addition of absolute acetone. The β -bromoallylmercaptomethylpenicillin assayed 2030 units per mg.

Analysis—Found, Br 17.81, C 36.80, H 3.76

γ -Chloroallylmercaptomethylpenicillin—30 gm. of γ -chloroallylmercaptoacetic acid (6) were added to a synthetic medium and the fermentation was conducted similarly to that described under *n*-propylmercaptomethylpenicillin. The tank was harvested after 86 hours, at which time the broth assayed 215 units per ml. A buffer of pH 6.2 was used with the ether and chloroform chromatographic columns. A large portion of the activity was found near the top of the ether column. When this fraction was applied to the chloroform column, a single large band was found near the center of the column. The sodium salt was prepared and dried as previously described. Attempts to crystallize this material have been unsuccessful. The amorphous penicillin assayed 1150 units per mg. and gave a differential assay value of 0.80.

Analysis— $C_{13}H_{16}ClN_2O_4S_2Na$. Calculated. Cl 9.17, N 7.25, S 16.58
Found. " 4.10, " 5.77, " 10.43

A sample was purified further by use of an ether-buffer (pH 6.2) chromatographic column. Although the activity was spread over a considerable portion of the column, differential assay values on various fractions gave similar values. Most of the active fractions were therefore combined and the penicillin was recovered. Analyses demonstrated that no further purification had been achieved. The separation on the column and the analyses are consistent with the interpretation that the new penicillin is mixed with a natural penicillin which gives a similar differential assay value.

n-Butylmercaptomethylpenicillin—Strain Q-176 was used with *n*-butylmercaptoacetic acid (7) (177 mg. per liter) and a medium similar to that described for cyclopentylmethylpenicillin. The ether-buffer (pH 6.2) chromatographic column contained a large band accounting for most of the units. When this fraction was chromatographed on a chloroform-buffer (pH 6.2) column, the activity was scattered over the lower two-thirds of the column and into the filtrates. Differential assays on various fractions gave similar values. Two separate sodium salts were prepared, one of the filtrate fractions and the other of the column fractions. These salts were crystallized separately by treatment with acetone, and were then recrystallized by solution in 90 per cent aqueous acetone, followed by addition of several parts of absolute acetone. Assay, differential assay, sulfur, and nitrogen determinations indicated that both fractions repre-

sented the desired *n*-butylmercaptomethylpenicillin. A small sample was recrystallized several times as described above. This sample assayed 3400 units per mg. and gave a differential assay value of 0.53.

Analysis— $C_{14}H_{21}N_2O_4S_2Na$. Calculated. N 7.60, S 17.40
Found. " 7.52, " 17.26

Isoamylmercaptomethylpenicillin—Isoamylmercaptoacetic acid (6) (194 mg. per liter) was used as the precursor with strain Q-176 and a medium similar to that described for cyclopentylmethylpenicillin. On the ether-buffer (pH 6.2) chromatographic column, the activity was spread over a large number of fractions in the filtrate and on the column. Differential assays indicated the presence of only one penicillin in all these fractions, with the exception of a small portion near the top of the column. These fractions were therefore combined for the chloroform-buffer (pH 6.5) column. Almost all of the activity was in the filtrate fractions of this column. The dried sodium salt which was recovered from these fractions crystallized upon treatment with absolute acetone. After recrystallization by solution in 5 parts of 90 per cent aqueous acetone followed by addition of absolute acetone, the penicillin assayed 2850 units per mg. and gave a differential assay of 0.51. After two additional recrystallizations the isoamylmercaptomethylpenicillin assayed 2900 units per mg. and gave a differential assay of 0.54.

Analysis— $C_{15}H_{23}N_2O_4S_2Na$. Calculated. N 7.33, S 16.76
Found. " 7.15, " 16.11

m-Trifluoromethylphenylmercaptomethylpenicillin—A corn steep medium and fermentation similar to those described for cyclopentylmethylpenicillin were employed. *m*-Trifluoromethylphenylmercaptoacetic acid (6) (280 mg. per liter) served as the precursor. When the penicillin was purified on an ether-buffer (pH 6.3) chromatographic column, a large band was found in the filtrate fractions. This material was further purified by use of a chloroform-buffer (pH 6.6) chromatographic column. The major portion was again found in the filtrate, although an appreciable amount of penicillin with a similar differential assay was held on the lower part of the column. The penicillin from the filtrate fractions was recovered as sodium salt and was crystallized by treatment with absolute acetone. The *m*-trifluoromethylphenylmercaptomethylpenicillin was recrystallized, first from 92 per cent aqueous acetone by addition of acetone, and then from water-*n*-butanol by evaporation *in vacuo* to remove the water. The resulting product assayed 1900 units per mg. and gave a differential assay

value of 0.36. The ultraviolet absorption curve demonstrated the presence of the *m*-trifluoromethylphenylmercapto group.³

Analysis— $C_{17}H_{18}F_3N_2O_4S_2Na$

Calculated. N 6.15, S 14.08, F 12.51

Found.⁵ " 6.13, " 13.73, " 12.47

γ -Phenylpropylmercaptomethylpenicillin— *γ -Phenylpropylmercaptoacetic acid* (6) (252 mg. per liter) was used as the precursor in a fermentation similar to that described for cyclopentylmethylpenicillin. On the ether-buffer (pH 6.3) chromatographic column the principal band was found in the filtrate fractions. This was also the case with the chloroform-buffer (pH 6.6) column which followed. The dried sodium salt that was prepared from this material was deeply colored, occasioning difficulty in the further purification. Crystallization took place following treatment with absolute acetone. After recrystallization from 90 per cent aqueous acetone by addition of absolute acetone, the material assayed 1100 units per mg. and gave a differential assay value of 0.54. As further recrystallization from aqueous acetone did not aid markedly in the purification, an effort was made to achieve the desired purification by recrystallization from water-butanol as described before. The resulting *γ -phenylpropylmercaptomethylpenicillin* assayed 1600 units per mg. and gave a differential assay value of 0.54. Although the material still contained colored impurities, further purification was not attempted.

Analysis— $C_{19}H_{23}N_2O_4S_2Na$. Calculated. N 6.51, S 14.89

Found. " 5.28, " 11.39

β -Phenoxyethylmercaptomethylpenicillin—254 mg. per liter of *β -phenoxyethylmercaptoacetic acid* (6) were added to a synthetic medium for fermentation in the manner described for *n*-propylmercaptomethylpenicillin. With a buffer of pH 6.4 on the ether-silica chromatographic column a large band was found on the top half of the column. This material separated into two bands of activity on a chloroform-buffer (pH 6.2) column. The band in the filtrate fractions proved to be the desired new penicillin. The dried sodium salt was crystallized by treatment with acetone. Recrystallization by solution in 89 per cent acetone followed by addition of absolute acetone yielded *β -phenoxyethylmercaptomethylpenicillin*, which assayed 1190 units per mg. and had a differential assay value of 0.84. The ultraviolet absorption curve demonstrated the presence of the desired acyl grouping in the penicillin.³

Analysis— $C_{19}H_{21}N_2O_4S_2Na$. Calculated. N 6.48, S 14.83

Found. " 6.41, " 14.62

⁵ Micro fluorine analysis performed by the Huffman Microanalytical Laboratories, Denver, Colorado.

β-Naphthylmercaptomethylpenicillin—N-(2-Hydroxyethyl)-*β*-naphthylmercaptoacetamide (6) (313 mg. per liter) was used as the precursor with strain Q-176. The procedure previously outlined for preparation of allylmercaptomethylpenicillin was followed. Marked loss was noted in the preparation of the sodium salt from amyl acetate. On the ether-buffer (pH 6.2)-silica column the activity was scattered over the column and into the filtrates. All active fractions were therefore combined for a chloroform-buffer (pH 6.2) column. Two bands were evident on this column. Both were investigated further. Only the lower band contained appreciable quantities of the desired penicillin. This fraction was further purified by use of a second chloroform column, this time with buffer of pH 6.4. 104,000 units of this material were transformed to the sodium salt and dried from the frozen state. The dried material assayed 625 units per mg. and gave a differential assay value of 0.89. On treatment with absolute acetone, crystallization occurred. After washing with absolute acetone, the dried material assayed 1230 units per mg. Following two successive recrystallizations from 80 per cent acetone with addition of absolute acetone, the *β*-naphthylmercaptomethylpenicillin assayed 2160 units per mg. and gave a differential assay value of 0.91. The ultraviolet absorption curve was in good agreement with that observed for the precursor.

Analysis— $C_{20}H_{19}N_2O_4S_2Na$. Calculated. N 6.39, S 14.62
Found. " 6.78, " 13.82

Phenylselenomethylpenicillin—Phenylselenoacetic acid (9) (258 mg. per liter) served as the precursor in a fermentation similar to that described for cyclopentylmethylpenicillin. The ether-buffer (pH 6.3) column contained a single band on the upper half of the column. The penicillin was purified further on a chloroform column with buffer at pH 6.3. The activity was spread over the lower two-thirds of the column. The active fractions were combined, and the sodium salt of the penicillin was prepared and dried. Treatment of the material (11.1 gm.) with absolute acetone gave a crystalline product. Recrystallization from 100 ml. of 87.5 per cent acetone by addition of 300 ml. of acetone yielded 7.0 gm. of phenylselenomethylpenicillin assaying 2400 units per mg. and giving a differential assay value of 0.74. A small sample was recrystallized again for analysis. It assayed 2660 units per mg. and gave a differential assay value of 0.74.

Analysis— $C_{18}H_{17}N_2O_4SSeNa$. Calculated. Se 18.14, N 6.44, S 7.36
Found. " 17.87, " 6.33, " 7.52

The ultraviolet absorption curve confirmed the presence of the desired penicillin.³

p-Methoxyphenoxymethylpenicillin—N-(2-Hydroxyethyl)-*p*-methoxyphenoxycetamide (6) (180 mg. per liter) was used as the precursor with strain NRRL 1976. It was noted that use of this compound caused a decrease of total penicillin yield. No effect on the growth of the mold was noticed. The ether-buffer (pH 6.4) silica column contained a band on the upper portion of the column. When this material was rechromatographed on a chloroform-buffer (pH 6.2) column, the activity was divided into two bands, one in the filtrate fractions and one on the column. The filtrate band was recovered in the usual fashion and was crystallized from acetone. After three recrystallizations from 90 per cent acetone by addition of acetone, the penicillin gave an ultraviolet absorption curve which was in good agreement with that of the precursor.³

Analysis— $C_{17}H_{19}N_2O_6SNa$. Calculated. OCH₃ 7.71, N 6.96
Found. " 7.07

After two more recrystallizations, the *p*-methoxyphenoxymethylpenicillin assayed 1120 units per mg. and gave a differential assay value of 0.92.

Found, OCH₃ 7.14, N 6.35

3-Thiophenemercaptomethylpenicillin—209 mg. per liter of 3-thiophenemercaptoacetic acid (6) were used as the precursor in a fermentation similar to that described for cyclopentylmethylpenicillin. A large portion of the activity on the ether-buffer (pH 6.2) column was found in a concentrated band near the top of the column. This material was converted into the sodium salt which was dried from the frozen state. Treatment with absolute acetone yielded a crystalline product which was twice recrystallized from 90 per cent acetone by addition of dry acetone. The sodium 3-thiophenemercaptomethylpenicillin assayed 2160 units per mg. and gave a differential assay value of 0.70.

Analysis— $C_{14}H_{16}N_2O_4S_2Na$. Calculated. N 7.10, S 24.38
Found. " 6.29, " 20.18

A sample was recrystallized from water-butanol in the manner previously described, and from aqueous acetone as described above. It assayed 2000 units per mg. and gave a differential assay value of 0.76.

Found, N 6.83, S 20.29

Experimental Procedure for Ultraviolet Absorption Data of New Biosynthetic Penicillins (2)³

The samples of penicillins and precursors for ultraviolet absorption spectra were weighed from lots on which analyses had been performed. They were dried *in vacuo* at 60° for 2 hours. About 1 mg. was weighed on a micro balance into a calibrated volumetric flask. All dilutions were made with calibrated volumetric ware. The penicillins were dissolved in 0.005 M

phosphate buffer, pH 7.0, and the absorption curve run against the same buffer solution as the blank. The precursors were dissolved in distilled water and run against distilled water as the blank.

The absorption data were obtained with a Beckman quartz spectrophotometer, with a hydrogen discharge lamp as a light source. Points were taken at 2 $m\mu$ intervals throughout the curves. The nominal band width obtained was less than 3 $m\mu$ between 220 and 360 $m\mu$, reaching a low value of 2 $m\mu$ band width.

The authors express their gratitude to Dr. G. H. A. Clowes, Dr. E. C. Kleiderer, and H. A. Shonle for their interest in this work.* The microanalyses were performed by W. L. Brown and H. L. Hunter. We are happy to acknowledge the work of G. L. Shaw in designing and installing the pilot plant equipment.

SUMMARY

Following the use of appropriate precursors some thirty new penicillins have been isolated and identified. In several instances the new penicillins are formed by the mold, with virtual exclusion of the natural penicillins. The side chains of these penicillins contain chemical groupings which may be considered as biologically foreign substances in that they are not normally found in natural penicillins. The activity of these penicillins, determined with *Staphylococcus aureus*, varies from about 550 to 3400 units per mg.

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* Valuable technical assistance has been given by Dorothea Huff, A. K. Keltch, Charleen W. McClain, Alberta Glanz, and Ruth Stewart.

THE EFFECT OF α -TOCOPHEROL ON THE UTILIZATION OF CAROTENE BY THE RAT*

BY R. M. JOHNSON† AND C. A. BAUMANN

(From the Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison)

(Received for publication, May 27, 1948)

In a previous study it was demonstrated that the ingestion of carotene by rats fed thiourea or thiouracil resulted in only negligible stores of vitamin A in the animal, but that normal stores accumulated when the effects of these drugs were corrected with thyroxine (1). The metabolic stimulants and depressants exerted only slight effects on vitamin A storage when the dietary source of the stored vitamin was vitamin A itself rather than carotene. These studies have now been extended to include another factor capable of influencing the rate of biological oxidations, *viz.* α -tocopherol.

The sparing effect of tocopherol on vitamin A and carotene *in vivo* was first described by Moore (2, 3), and the original observations were greatly amplified by Hickman and his associates (4-6). The latter workers observed, however, that, when a relatively large amount of tocopherol (5 mg. per rat daily) was fed to rats, growth due to carotene or vitamin A was less than when moderate amounts of tocopherol were fed. The effect was greater when carotene was fed. This suggested that tocopherol, like thiourea or thiouracil, might be capable of interfering with the conversion of carotene to vitamin A. In the present study relatively large amounts of tocopherol were administered to rats in various ways, and the storage of vitamin A was determined in rats fed either vitamin A itself or β -carotene. In addition attempts were made to associate differences in storage with the metabolic rate or the fecal excretion of carotene.

Methods

Weanling rats were placed on a diet low in vitamin A, consisting of casein 18, dextrin 65, cottonseed (Wesson) oil 5, brewers' yeast 8, and salts 4 (7). 16 drops of Drisdol¹ containing 4000 units of vitamin D were added per 10

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and the Jonathan Bowman Fund for Cancer Research.

† National Institute of Health Junior Research Fellow.

¹ A propylene glycol solution containing 10,000 U. S. P. units per gm. of a crystalline vitamin D from ergosterol; Winthrop Chemical Company, Inc., New York.

kilos of dist. The rats were maintained on this diet for 22 to 28 days, *i.e.*, until they failed to gain weight over a period of 7 days. They were then divided into two large series, comparable in weight and sex. One was fed 40 γ of β -carotene (8) per day; the other received 40 I.U. of vitamin A from the non-saponifiable portion of halibut liver oil. These series were further subdivided into groups of seven or eight animals, which received a daily supplement of 0 to 10 mg. of α -tocopherol² together with the vitamin A source, or 5 mg. of α -tocopherol 8 hours after the vitamin A source. The daily doses of vitamin A, β -carotene, or α -tocopherol were each fed in 3 drops of Wesson oil from calibrated droppers. Two groups also received α -tocopherol intraperitoneally. The tocopherol was prepared for injection by dissolving 1 gm. in 6 gm. of Tween 20³ and diluting to 50 ml. with water; 0.25 ml., containing 5 mg. of α -tocopherol, was injected intraperitoneally each day. All animals were given the appropriate supplements for 14 days and were killed by decapitation 24 hours after receiving the last supplement. The livers and kidneys were removed and analyzed for vitamin A as described previously (8, 9).

EXPERIMENTAL

Growth—The rats in all groups appeared to be in good health except for certain individuals in the groups that received Tween 20 and tocopherol intraperitoneally. Diarrhea was noticed in these rats and about three-fourths of them had adhesions between the liver and the diaphragm. The average weight gains for all the other groups were 49.5 gm. in 2 weeks for the series fed vitamin A and 52.0 gm. for those fed β -carotene. While there were some differences between groups within each series, there was no conclusive evidence of improved growth with increasing tocopherol intake (Table I), presumably because the amounts of carotene and vitamin A fed were more than adequate for such growth as was permitted by the other constituents of the diet and because the cottonseed oil in the basal diet supplied enough tocopherol (0.37 mg. per gm. of oil by the Furter-Meyer test) to stabilize the carotene or vitamin A in the digestive tract.

Storage of Vitamin A—When the source of vitamin A fed to the rats was a concentrate from halibut liver oil, similar amounts of vitamin A were found in the livers and kidneys of all groups, whether 0, 2.5, 5.0, or 10 mg. of α -tocopherol were fed with the concentrate or whether 5 mg. of α -tocopherol were fed 8 hours later. The average amounts found in these groups ranged from 52.5 to 61.2 γ of vitamin A (Table I). However, when β -caro-

² Synthetic *dl*- α -tocopherol purchased from Merck and Company, Inc., Rahway, New Jersey.

³ Sorbitan monolaurate polyoxyalkylene derivative, obtained from the Atlas Powder Company, Wilmington, Delaware.

tene was fed, the amounts of vitamin A found in the tissues varied inversely with the amount of α -tocopherol fed with the carotene. Rats receiving 0 to 2.5 mg. supplements of α -tocopherol stored 45.7 to 48.7 γ of vitamin A in their tissues. When 5 mg. of α -tocopherol were fed daily, this storage dropped to a mean of 31.9 ± 4.2 γ of vitamin A, and when 10 mg. of α -tocopherol were fed daily, only 25.7 γ of vitamin A were stored. However, when 5 mg. of α -tocopherol daily were fed 8 hours after the ingestion of the carotene, the amounts of vitamin A stored in the body were essentially

TABLE I

*Growth and Vitamin A Storage in Rats Fed Vitamin A or β -Carotene in Presence of Various Amounts of α -Tocopherol**

Daily dose of α -tocopherol	Vitamin A supplement†				β -Carotene supplement†			
	Weight gains	Kidney vitamin A	Liver vitamin A	Total vitamin A	Weight gains	Kidney vitamin A	Liver vitamin A	Total vitamin A
mg.	gm.	γ	γ	γ	gm.	γ	γ	γ
0	49.1	15.4	40.8	56.2 ± 1.8 ‡	48.6	16.7	30.5	47.2 ± 3.6
0.5	50.3	14.9	46.3	61.2 ± 2.7	45.0	17.4	31.1	48.7 ± 4.3
2.5	46.3	15.0	37.5	52.5 ± 1.3	51.0	15.9	29.8	45.7 ± 4.2
5	45.4	13.8	40.5	54.3 ± 1.8	50.0	14.9	17.0	31.9 ± 4.2
10	40.3	19.2	36.7	55.9 ± 2.5	63.5	19.1	6.6	25.7 ± 3.3
5§	46.4	11.1	36.8	47.9 ± 2.7	53.5	17.9	17.7	35.6 ± 5.1
5	68.4	20.6	34.7	55.3 ± 3.3	55.7	21.4	25.5	46.9 ± 4.0

* The figures are mean values with seven or eight rats per group.

† Supplements administered daily for 14 days.

‡ The standard error of the mean = $\sqrt{\sum(X - \bar{x})^2/n(n-1)}$, where X = individual values, $\bar{x}$ = the group mean, n = the number of individuals.

§ α -Tocopherol administered by daily intraperitoneal injections; see the text.

|| The vitamin A supplement was given in the morning and the α -tocopherol was fed approximately 8 hours later.

equal to those in the group not fed any supplement of tocopherol, 46.9 ± 4.0 γ versus 47.2 ± 3.6 γ . Incidentally, as in a previous study (8), significant amounts of vitamin A were found in the kidneys of all groups whether the dietary source of the vitamin was carotene or vitamin A itself (Table I). When the total amount in the body was low, kidney storage exceeded liver storage.

α -Tocopherol injected intraperitoneally appeared to depress vitamin A storage somewhat, but the significance of the result was doubtful since the condition of the animals was not always good. The rats injected intraperitoneally with 5.0 mg. of α -tocopherol contained 47.9 γ of vitamin A when the source of the vitamin was halibut liver oil, or about 15 per cent less than that found in comparable uninjected rats (Table I). In the series fed

β -carotene, the rats injected with tocopherol stored only 35.6 γ of vitamin A in the tissues as compared with 47.2 γ in the groups that received no tocopherol supplement, a reduction in vitamin storage of 25 per cent. Thus the effect of the injected tocopherol was somewhat greater in rats fed carotene than in those fed vitamin A.

Fecal Excretion of Carotene—In the previous experiments less vitamin A was stored when large amounts of α -tocopherol were fed with carotene than when carotene was fed alone, although the tocopherol did not interfere with the storage of preformed vitamin A. Thus, the deleterious effect of the tocopherol on the utilization of carotene must have been exerted either during the conversion of the carotene into vitamin A or prior to it, *e.g.*, by interfering with the absorption of carotene or by hastening its destruction. In an attempt to evaluate these possibilities, groups of rats (Table II)

TABLE II

*Fecal Excretion of β -Carotene in Presence of Varying Amounts of α -Tocopherol**

Level of α -tocopherol	No. of rats	Carotene excreted
mg.		per cent
0	6	45.7
0.5	4	45.4
5	3	46.2
10	9	48.2

* 44 γ of carotene were fed, together with the various levels of α -tocopherol in Wesson oil.

averaging 125 gm. in weight were fed the low vitamin A diet plus 0, 0.5, 5.0, or 10 mg. of α -tocopherol with single doses of 44 γ of β -carotene, and the feces were collected for 3 days thereafter. The amounts of carotene found in the feces were very uniform and represented 45.4 to 48.2 per cent of the amount ingested. The excretion of carotene by the rats fed the highest amount of tocopherol was no different from that by the group receiving no supplementary tocopherol (Table II). Thus the diminished storage of vitamin A could be attributed neither to an impaired absorption of carotene nor to any unusual destruction of the pigment in the digestive tract. The alternative remained, however, that tocopherol might have interfered with the conversion of carotene to vitamin A in the body.

Relation to Metabolic Rate—The O_2 consumption of tissues from vitamin E-deficient animals is known to be high (10–12), and hence it was of interest whether the doses of tocopherol that affected the utilization of carotene were capable of producing detectable changes in the metabolic rate of rats. During the course of the various feeding experiments described, certain rats

that had received 5 or 10 mg. of α -tocopherol for 10 to 12 days were fasted for 18 hours, placed in a metabolism apparatus (13, 14), and their consumption of O_2 measured. Five rats on the basal diet were found to consume 34.2 liters of O_2 per kilo per day as compared to 35.6 liters per kilo per day for four rats receiving 5 mg. of α -tocopherol daily and 38.6 liters per kilo per day for five rats on 10 mg. of the vitamin daily. In other words these amounts of tocopherol did not produce any measurable decreases in the O_2 consumption of the entire animal.

Nevertheless, specific oxidative processes may have been altered temporarily in certain tissues, such as, *e.g.*, the small intestine. Temporary alterations are suggested by the fact that 5 mg. of tocopherol failed to influence vitamin A storage when fed repeatedly 8 hours after the administration of carotene, although this amount of tocopherol exerted a marked effect when fed with the carotene. After several days of administration the amounts of tocopherol in most tissues of the body must have been high and essentially similar whether the tocopherol was fed separately or along with the carotene.

The two methods of administration, however, resulted in significantly different concentrations of tocopherol in the wall of the small intestine while carotene was being absorbed. The small intestines from rats killed 3 hours after the ingestion of 5 mg. of α -tocopherol averaged 392 γ of vitamin E by the Furter-Meyer test as compared to 176 γ 16 hours later. It has been suggested that carotene is converted to vitamin A in the small intestine (15-17) and preliminary evidence (18) indicates that at least the first step in the process could take place during absorption. Apparently, then, this reaction is disturbed only in the presence of the high concentrations of tocopherol reached while α -tocopherol is being absorbed, but which no longer obtain after the tocopherol is distributed among the other tissues of the body.

SUMMARY

1. Vitamin A and β -carotene were fed to depleted rats for 14 days. Certain of the animals also received 0 to 10 mg. of α -tocopherol daily with the vitamin A source, or 5 mg. of α -tocopherol 8 hours later. Colorimetric determinations were then made of the amounts of vitamin A stored in the livers and kidneys.

2. α -Tocopherol did not interfere with the storage of vitamin A when the vitamin itself was fed, but the stores of vitamin A due to β -carotene were lowered significantly when 5 or 10 mg. of α -tocopherol were fed with the carotene. Tocopherol injected intraperitoneally also appeared to interfere somewhat with the utilization of ingested carotene.

3. Tocopherol fed 8 hours after the carotene failed to interfere with the

storage of vitamin A. The fecal excretion of carotene was essentially the same in rats fed high levels of tocopherol as in control rats. The amounts of tocopherol fed did not alter the basal metabolic rates of the rats.

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THE ABSORPTION OF GLUTAMIC ACID AND GLUTAMINE*

By S. P. BESSMAN,† J. MAGNES,‡ PAULA SCHWERIN, AND
HEINRICH WAELSCH

(From the Departments of Biochemistry, New York State Psychiatric Institute and the
College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, April 15, 1948)

The occurrence of free glutamine in plant tissue extracts has been recognized for a considerable time, but its presence in the protein-free filtrates of mammalian tissues was established only recently (1). The functional significance of the free amide in tissues cannot be appreciated without a knowledge of its quantitative relationship to glutamic acid and of the biological mechanisms regulating the interconversions of the two compounds. A study of these questions was made possible by the development of a chemical micromethod for the determination of glutamic acid and glutamine, each in the presence of a large excess of the other (2, 3). The method was applied in studies of the absorption of glutamic acid, glutamine, and glutathione from the gut of the cat by analysis of the portal blood, and of the concentration changes of glutamic acid and glutamine in peripheral blood after oral administration of glutamic acid to human subjects. The analytical procedure was simplified and extended to permit the complete removal of glutathione. This modification became necessary not only for the use of the method in the experiments in which intestinal absorption of glutathione was studied, but also for its application to tissue analysis, which will be the subject of subsequent reports.

EXPERIMENTAL

Determination of Glutamic Acid and Glutamine—In the previous study on the glutamic acid and glutamine content of blood plasma and serum the glutamine concentration was calculated as the difference between total glutamic acid determined after acid hydrolysis in one sample and free glutamic acid determined in another. The method has been simplified, with a saving of material, by the direct determination of glutamine in the filtrate from the adsorption column. The filtrate (2 ml.) of the solution containing glutamic acid and glutamine and the wash water (2 ml.) were

* Supported by grants from the Rockefeller Foundation, the New York Foundation, and the Williams-Waterman Fund of the Research Corporation.

† Fellow of the Children's Hospital Research Foundation, Washington, D. C.

‡ On leave from the Department of Physiology, The Hebrew University, Jerusalem.

collected in a 10 ml. volumetric flask and hydrolyzed with 2 ml. of 6 N HCl for 1 hour. The hydrolysate was neutralized and diluted to 10 ml. as described previously. 2 ml. of the neutralized solution were taken for the glutamine determination. In pure solution and in the absence of asparagine the glutamic acid may be determined directly without further adsorption on a second column. Under such conditions the hydrolyzed solution was neutralized, 5 mm of acetic acid were added, and the solution was diluted to 10 ml. with water. 4 ml. of this solution were treated with ninhydrin. In all determinations on tissue filtrates the hydrolyzed solution, containing glutamic acid originating from glutamine, was passed through a second column. The recovery of glutamine alone or in solutions containing glutamic acid and amide, as obtained in the above procedure, amounted to 95 to 105 per cent.

Removal of Glutathione—In glutathione-containing solutions, cysteine and glutamic acid, equivalent to approximately 20 per cent of the tripeptide when expressed as glutamic acid, were liberated under the conditions employed for glutamine hydrolysis. The removal of glutathione by precipitation with metal salts (copper, cadmium, lead) led to considerable losses of glutamic acid. A nearly complete removal of glutathione or cysteine without loss of glutamic acid was accomplished by adsorbing the sulfhydryl compound on lead carbonate introduced on top of the aluminum oxide column. 1 mg. of lead carbonate ($2\text{PbCO}_3 \cdot \text{Pb}(\text{OH})_2$) suspended in 0.5 ml. of water was superimposed on the aluminum oxide column under gentle suction. By this modification of the column, glutathione and any cysteine were removed to the extent of at least 99 per cent (Table I). The last two glutathione experiments show that glutathione does not interfere with the direct determination of glutamine after glutamic acid adsorption. Glutathione in the amounts known to occur in mammalian tissue (4) can be successfully eliminated by this procedure.

Determination and Removal of Keto Acids—In the course of the investigation it became desirable to determine the keto acids formed after the intraintestinal administration of glutamic acid and glutamine. It was noted that high concentrations of keto acids, equivalent to more than 5 mg. of pyruvic acid per 100 ml. of plasma, lowered the glutamic acid recovery after acid hydrolysis of samples for the determination of total glutamic acid (glutamic acid plus glutamine) by the original method (3). No interference was experienced when the filtrates from glutamic acid adsorption, containing only glutamine, were submitted to acid hydrolysis, since most of the keto acid was retained in the column. If total glutamic acid is to be determined by the original procedure, the keto acids may be removed as the 2,4-dinitrophenylhydrazones, and they may be estimated by the same procedure.

In the experiments with blood plasma filtrates, 2 ml. of 0.1 per cent 2,4-dinitrophenylhydrazine in 2 N HCl were added to 1 ml. of the trichloroacetic acid filtrate. After 25 minutes the hydrazone was extracted with eight 4 ml. portions of benzene. The hydrazone was extracted from the benzene solution with three 2 ml. portions of a 10 per cent solution of sodium bicarbonate, the color was developed by the addition of 5 ml. of 2 N NaOH to 5 ml. of the extract, and read after 5 minutes in the Coleman junior spectrophotometer at wave-lengths 420 and 520 $m\mu$ in order to

TABLE I

Determination of Glutamic Acid and Glutamine in Presence of SH Compounds

SH compound added		In sample		Found	
		Glutamic acid	Glutamine	Glutamic acid	Glutamine
γ		γ	γ	γ	γ
Gluta-thione	604			4.7	
	1808			8.3	
	3012			14.9	
	564	41.4		41.8	
	600	37.3		37.3	
	1800	37.3		41.2	
	2400	37.3		45.8	
	1114	20.8	234*	21.9†	236
	2228	41.6	468*	45.1†	438
Cysteine	21.2	41.4		41.8	-

* Corrected for a content of 92.5 per cent of glutamine.

† Corrected for retention of 1 per cent of glutamine as glutamic acid (2).

determine ketoglutaric acid and pyruvic acid. Sodium pyruvate served as the standard (5, 6).

For the determination of the total glutamic acid content after the removal of keto acids, 1 ml. of the extracted trichloroacetic acid filtrate was hydrolyzed with 0.5 ml. of 6 N HCl at 100° for 1 hour, neutralized, and diluted to 5 ml. with water. 2 ml. aliquots were taken for the duplicate determinations.

Absorption Experiments. Cats—A cannula was introduced into the trachea of a cat (4 to 5 kilos) under diallylbarbituric acid¹ anesthesia, and heparin¹ was injected intravenously. After ligation of the gastrosplenic vein, a 2-way cannula with a side arm was introduced into the portal vein. Thus the portal blood flow was not obstructed. A blood sample (8 ml.) was removed before the injection into the small intestine of 5 ml. of saline

¹ We are indebted to Ciba Pharmaceutical Products, Inc., for a gift of diallylbarbituric acid and to Roche-Organon for a generous supply of heparin.

containing glutamic acid, glutamine, or glutathione, adjusted to pH 7.3. Two further blood samples were taken 15 and 30 minutes later. The cell volume was determined in all blood samples.

Man—Venous blood was taken from human subjects at least 18 hours after the last meal. Two further blood samples were removed 1 and 2 hours after the intake of 1 gm. of glutamic acid per 10 kilos of body weight. The whole amount of glutamic acid was suspended in 100 ml. of water.

RESULTS AND DISCUSSION

The analysis of the blood of the portal vein after the intrainestinal administration of glutamic acid or glutamine showed that both compounds

TABLE II

Concentration of Free Glutamic Acid and Glutamine in Blood Plasma of Portal Vein after Intrainestinal Administration of Glutamic Acid, Glutamine, and Glutathione

Values expressed as mg per 100 ml. of plasma.*

Time after admin- istration min	Cat 1		Cat 2		Cat 3		Cat 4		Cat 5		Cat 6	
	Acid	Amide	Acid	Amide	Acid	Amide	Acid	Amide	Acid	Amide	Acid	Amide
0	2.8	10.7	1.7	10.4	2.5	6.4	1.6	5.9	1.9	5.0	2.1	5.0
	100 mg glutamic acid administered				100 mg glutamine administered				205 mg glutathione administered			
15	12.9	4.2	6.6	8.3	3.9	12.7	1.9	12.3	1.9	5.0	1.8	5.6
30	14.8	13.0	1.0	15.5	3.1	25.8	1.6	8.8	1.7	5.0	2.3	5.4

* Per cent blood cells: Cat 1, 41, 36, 35; Cat 6, 49, 42, 41; Cats 2 to 5, change in blood cell volume less than 3 per cent.

passed the intestinal wall without any significant interconversion (Table II). A considerable increase of the administered compound occurred 15 minutes after administration, with only a small change in the level of the other. Simultaneously with the large increase in the glutamic acid concentration in the plasma after the administration of this amino acid (Cats 1 and 2) there was found, after 15 minutes, a decrease in the glutamine values. The increase of the glutamine concentration at 30 minutes may be interpreted as a release from the tissues of glutamine either formed from the administered glutamic acid or mobilized as a result of the increased glutamic acid concentration. Both phenomena, the decrease of the glutamine concentration and the following increase, have also been found in the peripheral blood plasma of human subjects after the ingestion of glutamic acid. The effect of an increased glutamic acid concentration in plasma in decreasing

the glutamine level appears to be part of a general mechanism since a lowering of the concentrations of glutamine, glycine, and residual amino nitrogen has been found under similar conditions in dogs (7).

In the glutamine experiments, there was found a small increase of glutamic acid, which is apparently not related to the glutamine concentration, and may therefore be due to a release of glutamic acid originating from the increased glutamine concentration in the tissue.

Glutamic acid and glutamine are apparently not converted into each other during the passage through the intestinal wall, and there seems to be no extensive deamination during this process. Only insignificant increases in the keto acid concentration were found and the optical-absorption ratios of the hydrazones at 420 and 520 $m\mu$ varied between 1.2 and 1.4, a result which indicates that the relative concentration of ketoglutaric acid did not change significantly, either during the absorption of glutamine or the parent amino acid.

After the intrainstestinal administration of an equivalent amount of glutathione, there was no change in the glutamic acid or glutamine concentration of the plasma of portal blood during the experimental period comparable with that found during the absorption of the amino acid and its amide. It has been pointed out in the experimental part that glutathione, if not removed, contributed to the glutamine fraction after acid hydrolysis about 20 per cent of its concentration in glutamic acid equivalents. Values obtained for glutamine in the glutathione experiments with and without the modification developed for the removal of glutathione agreed within the error of the method. It appears therefore that the tripeptide, if absorbed during the experimental period, is taken up rapidly by the cells or that it is not metabolized during passage through the intestinal wall to glutamic acid or glutamine to any considerable degree.

The direct evidence (8, 9) for the occurrence of glutamine and asparagine in proteins is based on the isolation of the amides from enzymatic hydrolysates. If changes in the glutamine content of the food proteins due to storage and preparation of the food are disregarded for the present, our experiments suggest the possibility that glutamic acid and glutamine are absorbed in about the ratio in which they occur in the original protein. Depending on the composition of the proteins ingested, varying amounts of the two compounds will therefore be absorbed and the organism, by enzymatic mechanisms, will have to adjust the amounts to the specific ratios of the tissues.

The oral administration of glutamic acid to human subjects led always to an increase of varying degree in the glutamic acid concentration of the peripheral blood (Table III). Two types of responses to the elevation of the blood glutamic acid may be distinguished. A small increase of the

glutamic acid concentration appeared to be accompanied by a considerable increase in the glutamine concentration, which may be interpreted as the return of the amidated amino acid from the tissue or a mobilization of tissue glutamine. A high glutamic acid concentration in the blood appeared to lead to a considerable decrease in the glutamine values 1 hour after the administration. In the experiments with cats, both the decrease and the increase in glutamine concentration were found in the portal blood of the same animal as a response to an elevated glutamic acid concentration. These findings are an additional demonstration of the influence of the blood concentration of one amino acid on that of another. These experiments do not permit any prediction as to the glutamic acid and glutamine levels in blood to be expected if glutamic acid is administered in combination with other amino acids, as in hydrolysates or whole protein.

Beneficial effects of the oral administration of glutamic acid to epileptics

TABLE III

Concentration of Free Glutamic Acid and Glutamine after Oral Administration of Glutamic Acid to Human Subjects (1 Gm. per 10 Kilos)

Values expressed as mg. per 100 ml. of plasma.

Time after admin- istration	Subject 1		Subject 2		Subject 3		Subject 4	
	Acid	Amide	Acid	Amide	Acid	Amide	Acid	Amide
<i>hrs.</i>								
0	0.6	8.4	0.6	10.6	1.2	8.4	0.8	10.3
1	1.0	10.3	1.2	14.0	5.0	5.8	9.5	8.3
2	1.0	8.9	0.8	11.3	0.7	6.3	1.0	10.0

and mental defectives have been reported (10, 11). It had been difficult to understand why a daily administration of only 10 to 20 gm. of glutamic acid could have any effect in view of the large amounts of glutamic acid ordinarily ingested with protein. The absorption experiments with the cat show that glutamine and glutamic acid may pass the intestinal tract without interconversion. Therefore, depending on the glutamine-glutamic acid ratio in the protein, a small amount of additional glutamic acid may considerably increase the relative intake of this amino acid. Our experiments with human subjects show that the ingestion of 2 or 3 times the therapeutic amount of glutamic acid leads to a significant increase in the blood glutamic acid level and to alterations in the metabolism of glutamine.

SUMMARY

A simplified modification of the method for the determination of glutamic acid and glutamine is described. Glutamine and glutamic acid are ab-

sorbed from the gut of the cat without interconversion. The elevated glutamic acid level in the portal blood is accompanied by a decrease in the glutamine level, followed by an increase. The oral administration of glutamic acid to human subjects leads to an increase of the glutamic acid level in the peripheral blood, with a simultaneous decrease or increase in the glutamine concentration.

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THE SPECTROPHOTOMETRIC ESTIMATION OF NICOTINE IN BLOOD*

By WILLIAM A. WOLFF, MARINA A. HAWKINS, AND W. E. GILES

(From the Tobacco Research Laboratory, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina)

(Received for publication, April 3, 1948)

Studies on tobacco smoking have been handicapped by the lack of methods sufficiently sensitive for estimating nicotine in blood. The amount of nicotine absorbed in the respiratory tract from cigarette smoke has been determined under conditions of normal smoking (2) and there are satisfactory procedures for the study of urinary excretion of nicotine (3, 4). About 50 γ of nicotine in a 1 hour specimen of dog urine was the smallest sample used in such studies (3). This quantity of nicotine is many times that to be expected in 10 ml. of blood from a smoker. With the study of blood nicotine levels as a general objective, the present authors undertook the development of a new analytical method which would permit the quantitative separation and estimation of the minute amount of nicotine present in a few ml. of blood from a smoker. By the use of several features, novel for nicotine methods, satisfactory techniques have been developed for handling 1 to 50 γ of nicotine in 10 to 20 ml. volumes of blood.

Apparatus and Reagents—

Distillation unit. An all-glass apparatus was constructed with parts connected by ball and socket or standard taper joints lubricated with a thin film of silicone grease. The distilling flask was made from a 500 ml. Kjeldahl type with a steam inlet tube fused into the neck and was fitted with a Corning No. 2020 connecting bulb.

Spectrophotometer. Beckman, model DU; used as a colorimeter.

Nicotine standards. Highest purity nicotine (Eastman, No. 1242) was made up in distilled water to a concentration of approximately 2 mg. per ml. and standardized with silicotungstic acid (5). This stock solution was diluted to give the desired concentration for working standards, usually 0.5 to 2 γ per ml.

Sodium chloride, C.P.

Sodium hydroxide, C.P. pellets.

Trichloroacetic acid. 50 per cent solution (weight by volume).

* This study was made possible by a grant from the Medical Relations Division of William Esty and Company, Inc., New York.

A preliminary report has been made (1).

Potassium acetate. c.p., 5.0 per cent in 95 per cent ethanol.

β -Naphthylamine. Eastman, No. 174; 1.2 per cent in 95 per cent ethanol.

Cyanogen bromide. Eastman, No. 919; 10.0 per cent in 95 per cent ethanol.

Ethanol. U. S. P. grade, redistilled from phosphoric acid and from solid sodium hydroxide.

Activated carbon. Nuchar C (West Virginia Pulp and Paper Company, New York).

TABLE I

Effect of Protein Precipitants on Recovery of Nicotine Added to Blood

40 γ of nicotine were added to each 10 ml. of blood.

Precipitant	Nicotine recovered
	γ
Copper tungstate*	7.9
	7.5
Zinc hydroxide	19.1
	20.0
Tungstic acid	20.4
	22.2
<i>m</i> -Phosphoric acid	36.0
	40.9
Trichloroacetic acid	38.6
	39.6

* Blood plasma used instead of whole blood.

EXPERIMENTAL

Critical studies have been made on numerous details used in nicotine methods. Selected experiments which have a bearing on the new features in the method presented here are summarized in the following sections.

Protein Precipitation—Extensive experience in this laboratory indicated that the direct determination of nicotine in blood was not feasible and, further, that nicotine must be separated quantitatively from blood protein. This requirement is a specific reagent which will precipitate the blood proteins and leave the same concentration of nicotine in both liquid and solid phases. This would permit analyses on aliquots of the protein-free filtrate. A number of protein precipitants were tested with blood or plasma containing definite amounts of added nicotine. The results given in Table I indicate that nearly 100 per cent of the added nicotine can be recovered by using trichloroacetic acid or *m*-phosphoric acid. Trichloroacetic acid was selected because of convenience in use and the relative stability of its solution in comparison to *m*-phosphoric acid.

Distillation—Nicotine distills very slowly under the conditions specified in the official method (5) and only a fraction of a 40 γ sample appears in the first 100 ml. of distillate. By increasing the alkali concentration to 5 M (20 per cent sodium hydroxide, weight by volume) and keeping the solution in the distillation flask saturated with sodium chloride, as suggested by Bowen and Barthel (6), 1 to 50 γ of nicotine is separated quantitatively in the first 20 ml. of distillate. Nicotine distills quite readily from alkaline solutions containing about 10 per cent ethanol, and much better recoveries of small samples are obtained than with the conventional steam distillation. All the nicotine is present in the first 20 ml. of ethanol-water distillate, thus permitting a larger aliquot (3 ml. instead of the usual 2 ml. of aqueous distillate) to be used in the subsequent colorimetric determination. Furthermore, the color is sharper and more stable with the ethanol distillates than with the steam distillates.

Colorimetric Estimation—Of the several colorimetric procedures recommended for the estimation of nicotine, the cyanogen bromide- β -naphthylamine reaction is sufficiently sensitive for the present purpose. Optimum conditions for color development with relatively large samples of pure nicotine in water solutions have been studied extensively (7, 8) with results which were confirmed in this laboratory. However, in the present study it was necessary to estimate much smaller quantities of nicotine, 0.05 to 1.0 γ , in the presence of interfering substances from blood. Our best results were obtained when the color was developed at 20–25° at about pH 9. The total volume was 4 ml. and contained 10 mg. of potassium acetate, 6 mg. of β -naphthylamine, 30 mg. of cyanogen bromide, and 2 ml. of ethanol. In this system the color reached a maximum intensity in 6 to 8 minutes after the reagents were added and faded thereafter. The rate of fading in the nicotine standard appeared to be different from that of the interfering substances. Consequently, readings were made at the time of maximum color intensity. The standard should contain approximately the same concentration of nicotine as the chromogenic material (blank plus nicotine) present in the unknown. If larger amounts of nicotine are used, 2 to 10 γ per determination, and the blank material does not contribute more than 10 per cent of the total color, satisfactory readings may be made at 20 to 30 minutes after the reagents are added, as recommended by others (7, 8).

Interfering Substances—Trichloroacetic acid or *m*-phosphoric acid filtrates from blood of non-smokers contain material which distills in steam from alkaline solution and reacts like nicotine with the cyanogen bromide- β -naphthylamine reagents. This material, designated the "nicotine blank," and probably a group of compounds, shows a marked increase following the ingestion of purine-containing beverages (tea and coffee) and

also following a diet high in protein. No increase resulted from a diet of fruit juices or from canned tomatoes. We were unable to reduce the blank value to zero by an exclusive carbohydrate diet for a 3 day period.

The blank material is remarkably stable in the presence of a variety of chemical reagents. There was no change in its value by (a) refluxing at 100° for 5 hours with either 10 per cent sodium hydroxide or 10 per cent phosphoric acid, (b) vigorous oxidation with hydrogen peroxide, potassium permanganate, or bromine water in acid solution at 20–25°, or (c) treatment with nitrous acid. Treatment with silver salts in the range pH 0 to 8 caused an apparent increase in color value; some 30 to 60 per cent of the material was precipitated by the silver salts.

The blank material shows the same solubility in organic solvents as does nicotine. It is readily extracted from alkaline aqueous solution by petroleum ether, benzene, toluene, ethyl ether, chloroform, ethylene dichloride, and carbon tetrachloride. Dilute acid, 0.1 N hydrochloric acid in water, removes the blank material from each of these organic solvents. Nicotine cannot be separated from the blank material by differential extraction with these solvents.

An attempt was made to differentiate nicotine from blank material by a spectrophotometric method. The absorption spectrum was determined for the reaction product of nicotine with the cyanogen bromide- β -naphthylamine reagents. Under the same conditions a concentrate of interfering substances prepared from blood of non-smokers was treated with cyanogen bromide- β -naphthylamine reagents and its absorption spectrum determined. Both spectra, Curves 1 and 2 in Fig. 1, showed a maximum absorption near 480 $m\mu$, with minor differences at other wave-lengths between 420 and 570 $m\mu$. From 570 to 1000 $m\mu$ there was no absorption. Below 420 $m\mu$ the spectra of both reaction products were nearly the same as that of the reagents (Curves 3, 4, and 5, Fig. 1). Differences between nicotine and the blank material are too small to permit the estimation of a ratio by reading at two wave-lengths.

As an indirect approach to the chemical nature of the "nicotine blank," the effect on the method was determined for a number of pure compounds. This was done by distilling with reagents plus the particular compound being tested and developing the color in the distillate. No effect was produced by trigonelline, nicotinic acid, creatine, methylamine, and tetramethylammonium hydroxide. A slight but negligible effect was produced by alanine, tyrosine, histidine, lysine, choline, ethanolamine, guanidine, and trimethylamine. Serious interference was caused by caffeine, theobromine, theophylline, and uric acid. Also, serious interference is caused by contact with rubber or rubber compounds.

In a series of adsorption tests it was found that the activated carbon.

Nuchar C, removed all the nicotine from aqueous solution but did not remove the blank material. The nicotine was completely removed by Nuchar C at all levels from pH 0 to 8. This seemed to effect a separation from the blank material. No method was found for eluting the nicotine from the carbon. Aliquots of filtrates from the blood of non-smokers were treated in three different ways to obtain the "nicotine blank." One aliquot was distilled directly, a second was treated with Nuchar C in the distilling flask before the addition of alkali and salt, and to a third were added 40 γ of nicotine, or 4 γ per ml. of the blood used, treated as the second aliquot. These findings, shown in Table II, suggested an indirect method for estimating nicotine in the presence of blank material. The

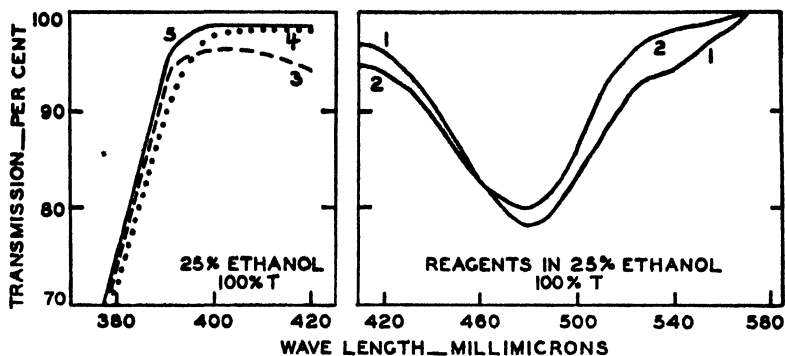


FIG. 1. Absorption spectra. Curves 1 and 5 for nicotine treated with cyanogen bromide- β -naphthylamine; Curves 2 and 3, same for "blank material" from blood of non-smoker; Curve 4 for reagents only.

total color material (true nicotine plus blank material) could be estimated colorimetrically by the cyanogen bromide- β -naphthylamine reaction. The solution could then be treated with Nuchar C, redistilled, and the blank material determined in the same way. The nicotine content would be the difference between the two determinations; *i.e.*, the amount of material adsorbed on the Nuchar C is assumed to be nicotine.

Procedure and Results

Lake 30 ml. of oxalated blood with 90 ml. of water and add, with stirring, 30 ml. of 50 per cent trichloroacetic acid. Stir vigorously for 5 minutes with a mechanical stirrer to produce a finely divided precipitate and filter through a dry paper (Whatman No. 1). Pipette 50 ml. of the filtrate into the distilling flask, and add 20 gm. of sodium chloride, 10 gm. of sodium hydroxide, and 5 ml. of ethanol. Connect the steam generator, which should be near the boiling point. Proceed with the distillation, adjusting

the Bunsen burner under the distilling flask so that a constant volume is maintained. Collect one 20 ml. fraction and one 15 ml. fraction of the distillate (add 5 ml. of ethanol to the second fraction and use as a check for the completeness of distillation in the first fraction). Pipette a second 50 ml. aliquot of the filtrate into another distilling flask (a separate apparatus is used for all distillations with Nuchar C), add about 0.5 gm. of

TABLE II

Effect of Nuchar C on Blank Material in Blood Filtrates from Non-Smokers and on Added Nicotine

Sample No.	Blank; direct distillation	Blank; distillation with Nuchar	Blank + 4 γ nicotine; distillation with Nuchar
	γ per ml.	γ per ml.	γ per ml.
1	0.25	0.20	
2	0.19	0.19	
3	0.14	0.14	
4	0.29	0.31	
5	0.20	0.22	
6	0.20	0.22	0.25
7	0.11	0.14	0.11
8	0.21	0.12	0.15
9	0.14	0.14	0.18
10	0.08	0.11	0.11

TABLE III

Estimation of Nicotine in Blood from Smokers with Use of Nuchar C

Subject No.	Total color material	Blank from Nuchar	Nicotine present
	γ per ml.	γ per ml.	γ per ml.
1	0.22	0.10	0.12
2	0.28	0.04	0.24
3	0.21	0	0.21
4	0.36	0.08	0.28
5	0.10	0	0.10
6	0.23	0.11	0.12

Nuchar C, the required amounts of sodium chloride, sodium hydroxide, and ethanol, and repeat the distillation as above. If a second aliquot of filtrate is not available, that portion of the distillate remaining after the color determination may be treated with Nuchar C and redistilled for an estimation of blank material.

To develop the color prepare a reagent blank, a standard, and portions of both distillates in dry test-tubes. To separate tubes add 3 ml. of 25 per cent ethanol, 0.2 to 2.0 γ of nicotine in 3 ml. of 25 per cent ethanol, 3

ml. of the first distillate, and 3 ml. of the second distillate. Add to each tube from micro burettes exactly 0.2 ml. of potassium acetate, 0.5 ml. of β -naphthylamine, and 0.3 ml. of cyanogen bromide, mixing after the addition of each reagent. Note the time and transfer to clean spectrophotometer cells. Read at 480 $m\mu$ 6 to 8 minutes after adding the reagents, setting the instrument at 100 per cent transmission with the reagent blank. The working temperature should be 20–22° and must not exceed 25°. Trustworthy results are obtained only by meticulous attention to details of spectrophotometric technique.

Recovery Experiments—More than 100 control determinations on nicotine in quantities of 1 to 50 γ added to 10 or 20 ml. of blood gave an average recovery of 90 to 95 per cent. With 1 γ the accuracy is limited somewhat by instrumental errors. With larger amounts, the error appears to be a percentage error rather than absolute.

Nicotine in Smokers' Blood—The method has been used for the determination of nicotine in blood drawn from smokers, with the results shown in Table III. Each blood was drawn at the end of an 8 hour period during which the subject smoked twenty or more standard brand cigarettes. The accuracy of these determinations is limited by the ratio of nicotine to the blank value. The nicotine level becomes somewhat uncertain if it is less than one-half the blank material. Within these limitations the method is suitable for the study of blood nicotine in relation to tobacco use.

SUMMARY

A method based on several new features has been developed for estimating 1 to 50 γ of nicotine in 10 to 20 ml. volumes of blood. This represents a 50-fold increase in sensitivity of nicotine methods and makes possible the study of blood nicotine in tobacco users.

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THE APPLICATION OF PEPTIDES CONTAINING β -ALANINE TO THE STUDY OF THE SPECIFICITY OF VARIOUS PEPTIDASES*

BY H. THEO HANSON† AND EMIL L. SMITH

*(From the Laboratory for the Study of Hereditary and Metabolic Disorders, and
the Departments of Medicine and Biological Chemistry, University of Utah
School of Medicine, Salt Lake City)*

(Received for publication, April 26, 1948)*

Very little is known about the rôle of β -alanine in biological processes, although it occurs in pantothenic acid, carnosine, and anserine. Its presence in the peptides is of interest since carnosine is the second most abundant nitrogenous extractive of muscle. Nevertheless, the function of these peptides is unknown, and β -alanine has not been found in proteins (2). In addition, the belief is widely held, on the basis of Abderhalden's studies (3), that peptides containing β -amino acids are not hydrolyzed by proteolytic enzymes. However, the histidine of carnosine is available for the growth of animals on a histidine-deficient diet (4).

The present investigation, the first of a series concerned with the behavior of peptides containing β -alanine, deals with the ability of certain peptidases to hydrolyze β -alanine peptides. It was found that leucine aminopeptidase does not distinguish between an α - and a β -amino group at the sensitive peptide bond. This situation is also likely to occur with other enzymes in which the main specificity is directed towards the amino acid moiety possessing the carboxyl group. This is probable with the aminopeptidase action of chymotrypsin and the amidase action of trypsin. It has recently been found by Schwert *et al.* (5) that trypsin hydrolyzes even ester linkages.

With enzymes which require a free amino group adjacent to the sensitive peptide bond, such as prolidase, glycyl-L-leucine dipeptidase, and glycyl-glycine dipeptidase, increasing the distance from the free amino group to the peptide bond decreases the sensitivity of the substrate quite markedly. In most instances, it is possible to give a quantitative statement of this effect.

It is evident that the specificity of peptidases towards α -amino acids is

* This investigation was aided by a grant from the United States Public Health Service.

A report of a part of this work was presented at a meeting of the American Society of Biological Chemists in March, 1948 (1).

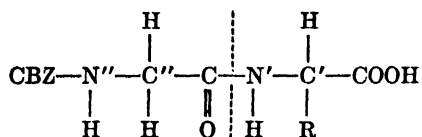
† Postdoctorate Fellow of the United States Public Health Service.

not absolute, and β -alanine-containing peptides can be used to secure information regarding enzyme specificity. It is apparent that the hydrolysis of compounds of unknown structure by proteolytic enzymes can no longer be regarded as proof that the compound contains only α -amino acids.

Carboxypeptidase

Carboxypeptidase was prepared from frozen beef pancreas by the method of Anson (6), and recrystallized five times as described by Neurath *et al.* (7). The relative sensitivity of the various compounds may be compared in terms of their proteolytic coefficients (C) since the hydrolysis follows the kinetics of a first order reaction (Table I). The most sensitive known substrate for carboxypeptidase, carbobenzoxyglycyl-L-phenylalanine (7, 8), is hydrolyzed about 800 times as fast as the β -alanine analogue, carbobenzoxy- β -alanyl-DL-phenylalanine. Likewise, carbobenzoxyglycyl-L-leucine is split about 1600 times as fast as carbobenzoxy- β -alanyl-L-leucine.

Carboxypeptidase hydrolyzes a variety of carbobenzoxy (CBZ) peptides of the accompanying configuration where the dotted line indicates



the point of cleavage. The introduction of a CH_2 group between N'' and C'' reduces the sensitivity of the substrate from 800 to 1600 times, as observed with the phenylalanine and leucine compounds. Although the second amino group has not usually been thought to influence the sensitivity of acylated peptides, this enzyme must have a strong affinity for N'' as well as for N' and the free COOH group. This is somewhat surprising since chloroacetyl-L-phenylalanine is rapidly hydrolyzed by carboxypeptidase (9). It must be concluded that an α substituent of some type (either Cl or an N-acylated residue) enhances the sensitivity of the substrate.

It is evident from the failure to observe splitting of carbobenzoxyglycyl- β -alanine that the intercalation of an additional CH_2 group between N' and C' reduces the sensitivity of the compound by at least 1000 times as compared to the corresponding L-alanine compound, or hydrolysis would have been noted at the enzyme concentration which was used.

Leucine Aminopeptidase

Highly purified leucine aminopeptidase from hog intestinal mucosa (10) rapidly splits L-leucylglycine (LG) and L-leucinamide (LA) with the kinetics of a first order reaction. It has now been found that L-leucyl- β -alanine

(LBA) is hydrolyzed just as rapidly as the amide and almost as rapidly as LG (Table II). The hydrolysis of LBA likewise follows first order kinetics and C is constant over a wide range of enzyme concentration.

The hydrolysis of LBA, like that of LA and LG, is greatly activated by

TABLE I
Action of Carboxypeptidase on Various Substrates

Substrates were present as sodium salts at a concentration of 0.05 M except for the racemic compound, which was used at 0.1 M. Incubations were carried out at 25°, buffered at pH 7.25 $\pm$ 0.25 with 0.066 M phosphate. C^1 is the proteolytic coefficient obtained by dividing the first order velocity constant (K) by the mg. of protein N per cc. of reaction mixture.

Substrate	Enzyme concentration	Time	Hydrolysis	C^1
	γ protein N per cc.	hrs.	per cent	
Carbobenzoxymethyl-L-phenylalanine	0.107			14
Carbobenzoxymethyl- β -alanyl-DL-phenylalanine	268	1.0	48	0.017
		1.25	59	0.019
		1.75	69	0.018
		2.0	73	0.017
	214	1.0	36	0.015
		1.75	54	0.015
		2.25	66	0.016
Carbobenzoxymethyl-L-leucine	2.1	0.75	42	2.4
		1.0	48	2.2
		1.25	53	2.1
		1.5	60	2.1
Carbobenzoxymethyl- β -alanyl-L-leucine	536	1.0	10	0.0014
		1.5	15	0.0015
		2.0	18	0.0014
		3.0	30	0.0016
		4.25	34	0.0013
		22	78	
Carbobenzoxymethyl-L-alanine*	40			0.038
Carbobenzoxymethylglycine*	320			0.0024
Carbobenzoxymethyl- β -alanine	320	19	0	
Carbobenzoxymethyl- β -alanyl- β -alanine	320	19	0	
Carbobenzoxymethyl- β -alanylglycine	320	19	4	

* Data of Stahmann, Fruton, and Bergmann (8).

Mn⁺⁺, and the activation by Mn⁺⁺ is a time reaction (10). Moreover, the finding that the rates of hydrolysis are not additive when both substrates are present (C_{LA} , 0.54; C_{LBA} , 0.51; C_{LA+LBA} , 0.62) also indicates that only one enzyme is involved; the small increase is probably due to the doubling of the substrate concentration.

The fact that this enzyme hydrolyzes LA, LBA, LG, and L-leucylglycylglycine at about the same rate suggests that its specificity is essentially that of an amidase, and that it is capable of hydrolyzing many types of substituted amides as well as peptides.

Aqueous extracts of rat muscle contain a leucine aminopeptidase which is activated by Mn^{++} and which rapidly hydrolyzes LG (11). This reaction follows zero order kinetics. LG is split by this extract 3 times as

TABLE II

Hydrolysis of L-Leucyl- β -alanine by Leucine Aminopeptidase of Hog Intestinal Mucosa

The purified enzyme was incubated at 40° and pH 8.0 for 3 hours with Mn^{++} before addition to the substrate. The hydrolytic tests were performed in veronal buffer at pH 8.0. The substrate concentration was 0.05 M except for the experiment involving two compounds in which each was present at 0.05 M.

Substrate	Enzyme concentration	Mn^{++} in test solution	Time	Hydrolysis	C ¹	C, average
	γ protein N per cc.	mole per l.	hrs.	per cent		
L-Leucylglycine	1.7	0.001				0.63
L-Leucinamide	42	None				0.014
"	3.4	0.001	1.0	21	0.55	
			1.5	29	0.49	
			2.0	39	0.55	
			2.5	47	0.55	
			3.0	52	0.54	
			4.0	64	0.56	0.54
L-Leucyl- β -alanine	42	None				0.038
"	1.7	0.001				0.52
"	3.4	0.001				0.50
"	4.2	0.001				0.50
"	8.4	0.001	0.75	35	0.51	
			1.0	46	0.54	
			1.25	60	0.52	0.52
L-Leucinamide + L-leucyl- β -alanine	3.4	0.001				0.62

fast as is LBA (Table III). The splitting of LBA also follows zero order kinetics and is activated by Mn^{++} .

Table III also gives the activities of an extract of human uterus (11). This preparation apparently contains a leucine aminopeptidase which is activated by Mn^{++} and another enzyme which is not so activated but which also acts on LG. In the presence of Mn^{++} , this extract hydrolyzes LG much more rapidly than LA; the second enzyme which acts on LG is probably not homospecific with the intestinal leucine aminopeptidase (11).

The hydrolysis of LG by the uterine preparation proceeds about 8 times as fast as the hydrolysis of LBA as judged from the zero order coefficients. The splitting of LBA is activated by Mn^{++} .

If the leucine aminopeptidases of different origin were completely homo-

TABLE III

Action of Leucine Aminopeptidase of Rat Muscle and of Human Uterus on L-Leucyl- β -alanine

The rat muscle extract was a freshly prepared aqueous solution. The uterine extract was prepared from an acetone-dried powder of an aqueous extract of the tissue. The experiments were performed at 40° in veronal buffer at pH 8.0. C^0 is the zero order velocity constant expressed as per cent hydrolysis per minute per mg. of protein N per cc.

Tissue	Substrate	Mn^{++}	Enzyme concentration	Time	Hydrolysis	C^0
				hrs.	per cent	
Rat muscle	L-Leucylglycine	0.001	0.30	0.5	24	2.7
				1.0	43	2.4
				1.5	64	2.4
				2.0	83	2.3
	L-Leucyl- β -alanine	0.001	1.20	0.5	28	0.78
				1.0	58	0.76
				1.5	82	0.76
				3.0	96	
	L-Leucyl- β -alanine	None	1.20	1.0	20	0.27
				2.0	44	0.31
				3.0	70	0.38
Human uterus	L-Leucylglycine	0.001	0.0692	1.0	22	5.3
				1.5	29	4.6
				2.0	41	4.9
				2.5	52	4.9
				3.0	62	4.9
	L-Leucyl- β -alanine	0.001	0.692	1.0	31	0.74
				1.5	40	0.63
				2.0	52	0.62
				2.5	55	0.52
				3.0	76	0.60

specific enzymes, the ratio $C_{LG}:C_{LBA}$ should be constant. However, with the preparation from hog intestine the ratio is 1.2, with rat muscle 3.1, and with human uterus 8.1. This is additional evidence for the presence of a second enzyme in uterus which acts on LG and which possesses a specificity different from that of the aminopeptidase.

Prolidase

A partially purified preparation of prolidase from hog intestinal mucosa (10) hydrolyzes glycyl-L-proline (GP) about 330 times more rapidly than β -alanyl-L-proline (BP) (Table IV). The hydrolysis of both compounds proceeds with the kinetics of a first order reaction. The splitting of BP is activated by Mn^{++} , and the activation is a time reaction (11). That both

TABLE IV

Action of Prolidase on β -Alanyl-L-proline

In each instance, the enzyme was incubated at 40° and pH 8.0 with Mn^{++} for 3 hours before addition to the test solutions. The tests were performed in veronal buffer at pH 8.0 at a final Mn^{++} concentration of 0.001 M. When two compounds were used, each was present at 0.05 M. C^1 is the first order proteolytic coefficient.

Preparation	Substrate	Enzyme concentration	Time	Hydrolysis	$C^1 \times 10^3$	C , average $\times 10^3$
		mg. protein N per cc.	hrs.	per cent		
Hog intestinal Mucosa I	Glycyl-L-proline	0.0039				360
	β -Alanyl-L-proline	1.16	1.75	28	1.2	
			2.25	37	1.3	
			6.0	60	0.9	
			8.0	75	1.1	
Hog intestinal Mucosa II	Glycyl-L-proline	0.0016				1020
	Glycyl-L-proline + β -alanyl-L-proline	0.0016				450
Rabbit muscle	Glycyl-L-proline	0.31	1.0	44	14	13.3
			1.5	60	14	
			2.0	64	12	
			19	24	0.057	
			25.5	29	0.053	
	β -Alanyl-L-proline	1.84	43	52	0.068	0.062
			48	53	0.062	
			0.5	12	6.1	
			1.0	24	6.5	
			1.5	32	6.0	
	Glycyl-L-proline + β -alanyl-L-proline	0.31	2.0	44	6.8	6.4

compounds appear to be split by the same enzyme is also indicated by experiments in which both were present simultaneously but with an enzyme concentration which was too low to effect any hydrolysis of BP. Under these circumstances, the rate of splitting of GP was reduced by 56 per cent. This suggests that the two compounds compete for the same active center in the enzyme.

Prolidase of rabbit muscle is homospesific with the intestinal enzyme (11). The homospesificity is further indicated by the fact that GP is

hydrolyzed about 210 times faster than BP in experiments with the enzyme from muscle. The inhibition in a competition experiment was 52 per cent.

The great reduction in the rate of hydrolysis by the insertion of a CH_2 group between the free amino group and the sensitive peptide bond indicates that this distance is quite critical. Nevertheless, BP can combine equally well with the enzyme, as is indicated by the 50 per cent inhibition which occurs when both compounds are present. These experiments

TABLE V

Hydrolysis of Glycyl-L-leucine and β -Alanyl-L-leucine by Uterine Extract

The extract of human uterus was tested at 40° in the presence of 0.001 M Zn^{++} and 0.1 M phosphate buffer at pH 7.8. The single substrates were present at a concentration of 0.05 M . In the mixture, each substrate was used at a concentration of 0.05 M . C^1 is the proteolytic coefficient obtained from the first order velocity constant, K , and the enzyme concentration, E , expressed in mg. of protein N per cc. where $C^1 = K/E$.

Substrate	Enzyme concentration	Time	Hydrolysis	C^1
	mg. protein N per cc.	hrs.	per cent	
Glycyl-L-leucine	0.0102	1.0	34	0.30
		1.5	51	0.34
		2.0	64	0.36
		2.5	75	0.39
		3.0	80	0.38
β -Alanyl-L-leucine	0.346	1.5	12	0.0018
		2.0	14	0.0015
		2.5	16	0.0014
		3.25	22	0.0017
Glycyl-L-leucine + β -alanyl-L-leucine	0.0102	1.0	28	0.23
		1.5	36	0.21
		2.0	45	0.21
		2.5	50	0.20
		3.0	66	0.26

provide further evidence for the necessity of a free amino group in the substrates for this enzyme. It has previously been reported that carbobenzoxyglycyl-L-proline (12) and carbobenzoxyglycylhydroxy-L-proline (10) are resistant to hydrolysis.

Glycyl-L-leucine Dipeptidase

A dipeptidase which acts on glycyl-L-leucine has recently been described (13). The enzyme from human uterus which is activated by Zn^{++} hydrolyzes glycyl-L-leucine about 250 times more rapidly than β -alanyl-L-leucine (Table V). The hydrolysis follows the kinetics of a first order reaction.

The presence of the β -alanine peptide inhibits the hydrolysis of glycyl-L-leucine about 35 per cent, as is indicated by a reduction of C from 0.36 to 0.22. Under the conditions employed for this test, splitting of β -alanyl-L-leucine would be negligible. This demonstrates that the substrates compete for the same active center. The situation here is analogous to that already discussed for prolidase.

Glycylglycine Dipeptidase

The properties of a glycylglycine dipeptidase which is activated by Co^{++} have recently been described (14). It has now been found that

TABLE VI

Action of Tissue Extracts on Dipeptides Containing β -Alanine

The tissue extracts were similar to those described in Table III. The tests were performed at 40° in veronal buffer at pH 7.8.

Tissue	Substrate	Enzyme concentration	Time	Hydrolysis		
				No metal	0.001 M Co^{++}	0.001 M Mn^{++}
				per cent	per cent	per cent
Human uterus	Glycyl- β -alanine	0.222	24	17	25	12
	β -Alanylglycine	0.346	24	0	1	1
	β -Alanyl- β -alanine	0.346	24	3	2	-1
	Glycylglycine	0.069	3	40	94	
	Glycyl-DL-alanine	0.222	1.5	74	78	87
Rat muscle	Glycyl- β -alanine	0.16	24	9	11	7
	β -Alanylglycine	0.56	43	2	1	0
	β -Alanyl- β -alanine	0.56	43	0	-1	1
	Glycylglycine	0.16	6	19	100	63

extracts rich in this enzyme do not split β -alanylglycine or β -alanyl- β -alanine (Table VI). It is also evident that glycyl- β -alanine is slowly hydrolyzed, and that the reaction may be slightly activated by Co^{++} .

With the uterine preparation, the hydrolysis of glycyl-DL-alanine is slightly activated by Mn^{++} and not by Co^{++} . It is evident that the splitting of glycyl- β -alanine is not due to this enzyme. The fact that β -alanyl- β -alanine and β -alanylglycine are not hydrolyzed also indicates that the enzyme which acts on glycyl-DL-alanine has no action on these β -alanine peptides.

Tripeptidase Activity of Rat Muscle

A fresh extract of rat muscle hydrolyzes triglycine very rapidly, and acts on glycyl- β -alanylglycine and diglycyl- β -alanine (Table VII). The

hydrolysis of β -alanylglycylglycine was very slow. The aged extract hydrolyzes only one peptide bond of triglycine; this is consistent with the known lability of the glycylglycine dipeptidase of this tissue (14).

The loss of activity towards β -alanylglycylglycine in the aged extract indicates that the splitting of this compound is due to an enzyme distinct from the one which acts on triglycine. Whether the other three tripeptides are all split by the same enzyme cannot be decided at present.

EXPERIMENTAL

The tests were performed in 2.5 cc. volumetric flasks. Hydrolysis was followed on 0.2 cc. samples by the titration of liberated carboxyl groups

TABLE VII

Action of Rat Muscle Extract on Tripeptides Containing β -Alanine

A crude filtered aqueous extract of rat muscle was used for these tests within a few hours after the animal was killed. The aged extract had been allowed to stand in the refrigerator for 3 days. The enzyme concentration was 0.60 mg. of protein N per cc. in the test solutions. The experiments were performed at 40° in veronal buffer at pH 7.8.

Substrate	Time	Hydrolysis	
		Fresh extract	Aged extract
	hrs.	per cent	per cent
Triglycine	3	77	76
	20	147*	- 102
Glycyl- β -alanylglycine	3	47	48
	20	106	100
Diglycyl- β -alanine	3	16	22
	20	52	56
β -Alanylglycylglycine	3	7	3
	20	19	7

* Hydrolysis of a single peptide bond is given as 100 per cent.

(15). The splitting is expressed as 100 per cent for the complete hydrolysis of one peptide bond. Except where otherwise noted, the substrate concentration was 0.05 M.

Rabbit muscle prolidase was prepared from a crude, filtered, aqueous extract of fresh muscle. The precipitate obtained by the addition of 2 volumes of cold acetone to the extract was collected, washed with cold acetone, and dried. This powder was extracted with water, and the precipitate which formed between 0.4 and 0.6 saturation with ammonium sulfate was dialyzed and filtered.

In addition to the compounds used in the enzymatic studies, the synthe-

sis of a number of other derivatives of β -alanine and of two derivatives of L-alanine is described.

Glycyl- β -alanine

Carbobenzoxyglycyl- β -alanine—To a solution at 0° of 4 gm. of β -alanine in 20 cc. of 2 M NaOH, there were added with cooling and shaking an additional 25 cc. of 2 M NaOH and an ethereal solution of carbobenzoxyglycyl chloride from 12.6 gm. of carbobenzoxyglycine. After standing for 1 hour at room temperature, the ether layer was discarded and the aqueous phase was acidified to Congo red with 5 M HCl. Yield, 8.5 gm.; rosettes of needles. After recrystallization from hot water, the melting point was 140°.

$C_{13}H_{17}O_5N_2$.	Calculated.	C 55.7, H 5.8, N 10.0
280.3	Found.	" 55.8, " 5.8, " 10.1

Chloroacetyl- β -alanine—To 15 gm. of β -alanine in 42 cc. of 4 M NaOH at 0°, there were added alternately in portions, with cooling and shaking, 21 gm. of chloroacetyl chloride in 85 cc. of ether and 65 cc. of 4 M NaOH. After standing for 1 hour at room temperature, with occasional shaking, the aqueous solution was acidified. The solvent was evaporated *in vacuo*, and the residue extracted three times with hot acetone. The combined acetone solutions were concentrated under reduced pressure until precipitation began. Crystallization was completed by the addition of chloroform. Yield, 21 gm. The compound was recrystallized from chloroform as plates, with a melting point of 95°. This compound has been described as an oil (16).

$C_5H_8O_2NCl$ (165.6). Calculated, N 8.5; found, N 8.6

Glycyl- β -alanine—16 gm. of chloroacetyl- β -alanine were allowed to stand for 3 days with 80 cc. of concentrated aqueous NH_4OH . The compound crystallized on addition of ethanol after concentration *in vacuo*. The crystals were extracted three times with hot 95 per cent ethanol, and recrystallized twice from water-ethanol. Yield, 8.1 gm. of small square plates; m.p., 228° (decomposition). Miyamichi (16) gives 233° (decomposition); Abderhalden and Reich (3) give 230° (decomposition).

$C_6H_{10}O_3N_2$.	Calculated.	C 41.1, H 6.9, N 19.2
146.1	Found.	" 41.3, " 7.1, " 19.3

This compound was also obtained in quantitative yield by hydrogenation of carbobenzoxyglycyl- β -alanine.

β -Alanyl- β -alanine

Carbobenzoxy- β -alanyl- β -alanine—4.3 gm. of β -alanine were coupled as described above with the acid chloride from 10 gm. of carbobenzoxy- β -

alanine (17). Acidification gave 5.6 gm. of needles. These were recrystallized from hot water; m.p., 144–145°.

$C_{14}H_{18}O_4N_2$ (294.9). Calculated, N 9.52; found, N 9.59

β -Alanyl- β -alanine—3 gm. of carbobenzoxy- β -alanyl- β -alanine were dissolved in 50 cc. of methanol containing 3 cc. of water and 3 cc. of glacial acetic acid. The compound was hydrogenated at atmospheric pressure in the presence of palladium black. After filtration, the solution was concentrated *in vacuo*, and the dipeptide crystallized as tiny needles on standing with ethanol. Yield, 1.5 gm.; m.p., 212°.

$C_8H_{12}O_3N_2$ (160.2). Calculated, N 17.49; found, N 17.44

β -Alanylglycine

Carbobenzoxy- β -alanylglycine—4 gm. of glycine were coupled with the acid chloride from 5 gm. of carbobenzoxy- β -alanine as described above. Yield, 6.7 gm. of needles. After recrystallization from hot water, the melting point was 146–149°.

$C_{13}H_{17}O_4N_2$. Calculated. C 55.7, H 5.8, N 10.0
280.3 Found. " 56.0, " 5.9, " 10.1

β -Alanylglycine—4.0 gm. of carbobenzoxy- β -alanylglycine were hydrogenated as described above. Yield, 2.0 gm. of prisms on standing with ethanol; m.p. 226°.

$C_8H_{10}O_3N_2$ (146.1). Calculated, N 19.19; found, N 19.35

β -Alanyl-L-leucine

Carbobenzoxy- β -alanyl-L-leucine—6.5 gm. of L-leucine were coupled with the acid chloride from 5 gm. of carbobenzoxy- β -alanine as described above. After acidification, the product crystallized as tiny plates. After recrystallization from hot water, the yield was 7.2 gm.; m.p., 111°.

$C_{17}H_{24}O_4N_2$. Calculated. C 60.7, H 7.2, N 8.3
336.4 Found. " 60.6, " 7.3, " 8.5

β -Alanyl-L-leucine—Hydrogenation of 2.6 gm. of the above compound gave 1.4 gm. of needles on standing with ethanol; m.p., 245°.

$C_9H_{14}O_3N_2$ (202.2). Calculated. N 13.9; found, N 14.2
[α]_D²⁵ = -31.0° (1.5 % in water)

L-Leucyl- β -alanine—8 gm. of carbobenzoxy-L-leucine hydrazide were converted to the azide as previously described (18). The washed and dried ether solution of the azide was added to an ethyl acetate solution of β -alanine ethyl ester (from 5.5 gm. of the hydrochloride). The mixture was allowed to stand overnight. It was then washed with dilute HCl,

water, dilute bicarbonate, and water. The solution was dried over Na_2SO_4 and concentrated to a thick syrup. The carbobenzoxy dipeptide ester was saponified in 50 cc. of methanol by the addition of 20 cc. of 1 M NaOH. After 30 minutes at room temperature, the solution was acidified and concentrated to dryness. The oily carbobenzoxy dipeptide was hydrogenated in the usual manner. The peptide crystallized from 95 per cent ethanol; m.p., 214° . The yield was 2.8 gm., or 49 per cent of theory calculated from the quantity of hydrazide.

$\text{C}_8\text{H}_{14}\text{O}_5\text{N}_2$ (202.2). Calculated, N 13.85; found, N 13.87
 $[\alpha]_D^{25} = +28.0^\circ$ (5 % in water)

DL-Leucyl- β -alanine has been described by Abderhalden and Reich (3).

β -Alanyl-L-proline

Carbobenzoxy- β -alanyl-L-proline—5.7 gm. of L-proline were coupled with carbobenzoxy- β -alanyl chloride as described above. After acidification, the oily product was extracted into ethyl acetate and dried over Na_2SO_4 . 7.1 gm. of needles were obtained after cautious addition of dry petroleum ether; m.p., $91-93^\circ$.

$\text{C}_{16}\text{H}_{20}\text{O}_6\text{N}_2$ (320.3). Calculated, N 8.75; found, N 8.69

β -Alanyl-L-proline—Hydrogenation of 5 gm. of the above compound gave 2.4 gm. of prisms on standing with ethanol; m.p., 211° .

$\text{C}_8\text{H}_{14}\text{O}_5\text{N}_2$ (186.2). Calculated, N 15.05; found, N 14.77
 $[\alpha]_D^{25} = -93.3^\circ$ (1.5 % in water)

Carbobenzoxy- β -alanyl-DL-phenylalanine

Carbobenzoxy- β -alanyl-DL-phenylalanine Ethyl Ester—To an ethereal solution of phenylalanine ethyl ester (from 6.4 gm. of the hydrochloride), there was added at 0° a 10 per cent excess of carbobenzoxy- β -alanyl chloride. This was shaken with a cold saturated aqueous solution of potassium bicarbonate. Ethyl acetate was added to the reaction mixture to dissolve the carbobenzoxy dipeptide ester which crystallized spontaneously. After 1 hour at room temperature, the ethyl acetate-ether solution was washed and dried in the usual manner. 8.5 gm. of prisms were obtained by concentrating the solution *in vacuo*. After recrystallization from ethanol-water, the melting point was $88-89^\circ$.

$\text{C}_{22}\text{H}_{26}\text{O}_5\text{N}_2$ (398.5). Calculated, N 7.20; found, N 7.17

Carbobenzoxy- β -alanyl-DL-phenylalanine—3.4 gm. of the above ester were saponified at room temperature in 30 cc. of ethanol and 9.2 cc. of M NaOH. After 30 minutes, the solution was acidified to yield 2.3 gm. of needles; m.p., 142° .

$C_{20}H_{22}O_4N_2$ (370.4). Calculated, N 7.57; found, N 7.62

β -Alanylglycylglycine

Carbobenzoxy- β -alanylglycylglycine—The chloride obtained from 12 gm. of carbobenzoxy- β -alanine was coupled to glycylglycine prepared from 5.7 gm. of glycine anhydride (19). Yield, 14 gm. The compound was recrystallized from methanol, and then from water; m.p., 184–185°.

$C_{18}H_{19}O_6N_3$ (337.3). Calculated, N 12.5; found, N 12.8

β -Alanylglycylglycine—5.4 gm. of the carbobenzoxy tripeptide were hydrogenated and worked up in the usual manner. The tripeptide was recrystallized from water-ethanol. Yield, 3.1 gm.; m.p., 228° (decomposition).

$C_7H_{13}O_4N_2$ (203.2). Calculated, N 20.7; found, N 21.0

Glycyl- β -alanylglycine

Carbobenzoxyglycyl- β -alanylglycine—2.4 gm. of β -alanylglycine were coupled with 4.1 gm. of carbobenzoxyglycyl chloride. Yield, 4.6 gm. in rosettes of small needles. The melting point was 177–180° after recrystallization from hot water.

$C_{18}H_{19}O_6N_3$ (337.3). Calculated, N 12.5; found, N 12.7

Glycyl- β -alanylglycine—3 gm. of the above compound gave on hydrogenation 1.6 gm. of the tripeptide; m.p., 230° (decomposition).-

$C_7H_{13}O_4N_2$ (203.2). Calculated, N 20.7; found, N 20.9

Diglycyl- β -alanine

Carbobenzoxydiglycyl- β -alanine—7.6 gm. of carbobenzoxyglycyl chloride were coupled with 4.5 gm. of glycyl- β -alanine as described above. Yield, 7.0 gm.; m.p., 187–189°, after recrystallization from hot water.

$C_{18}H_{19}O_6N_3$ (337.3). Calculated, N 12.5; found, N 12.5

This compound was also prepared by saponification of the ethyl ester described below. Calculated, N 12.5; found, 12.4.

Carbobenzoxydiglycyl- β -alanine Ethyl Ester—7.5 gm. of carbobenzoxyglycylglycine hydrazide were converted to the crystalline azide (20). The azide was coupled in ethyl acetate with β -alanine ester prepared from 3.1 gm. of the hydrochloride. After 36 hours at room temperature, the solution was worked up in the usual manner and concentrated *in vacuo*. Yield, 3.9 gm.; m.p., 142°, after recrystallization from ethyl acetate-petroleum ether.

$C_{17}H_{21}O_4N_3$. Calculated. C 55.9, H 6.3, N 11.5
365.4 Found. " 56.1, " 6.6, " 11.4

Diglycyl- β -alanine—5.2 gm. of carbobenzoxydiglycyl- β -alanine were hydrogenated as usual. Water was added to dissolve the product before the catalyst was filtered off. The tripeptide crystallized on removal of the solvent. Yield, 3.0 gm.; m.p., 200° (decomposition).

$C_7H_{13}O_4N_3$ (203.2). Calculated, N 20.7; found, N 20.8

Carbobenzoxy- β -alanyl-glycine Ethyl Ester—The acid chloride from 12 gm. of carbobenzoxy- β -alanine was coupled in ethyl acetate with glycine ethyl ester (from 14 gm. of the hydrochloride). The solution was allowed to stand for several hours, the crystalline ester hydrochloride was filtered off, and the filtrate was washed and dried as usual. Evaporation of the solvent gave 6.8 gm. of the product; m.p., 95–96°.

$C_{15}H_{21}O_6N_3$ (308.2). Calculated, N 9.1; found, N 9.0

Carbobenzoxy- β -alanyl-glycinamide—6.5 gm. of the above ester were dissolved in 200 cc. of absolute methanol which had been saturated in the cold with NH_3 gas. After standing for 2 days at room temperature, the solution was repeatedly concentrated *in vacuo* with methanol to yield 4.8 gm. of crystals; m.p., 176° after recrystallization from hot water.

$C_{15}H_{17}O_4N_3$. Calculated. C 55.9, H 6.1, N 15.1
279.3 Found. " 56.1, " 6.5, " 15.3

β -Alanyl-glycinamide Acetate—3 gm. of the above compound were hydrogenated in the usual way. The product was recrystallized from methanol-ether. Yield, 1.4 gm.; m.p., 118–120°.

$C_{17}H_{19}O_4N_3$ (205.2). Calculated, N 20.5; found, N 20.2

Carbobenzoxyglycyl- β -alanine Ethyl Ester—An ethereal solution of the chloride from 9 gm. of carbobenzoxyglycine was added at 0° to an ethyl acetate solution of β -alanine ester prepared from 4.3 gm. of the hydrochloride. The solution was shaken with dilute bicarbonate for 20 minutes. The ethyl acetate-ether layer was worked up in the usual manner. The product crystallized in rosettes of needles after standing in ether-petroleum ether. Yield, 3.5 gm.; m.p., 63–64°.

$C_{15}H_{21}O_6N_3$ (308.2). Calculated, N 9.1; found, N 8.8

Carbobenzoxyglycyl- β -alaninamide—3.1 gm. of the above ester were amidated as described above. On concentration *in vacuo*, 2.3 gm. of fine needles were obtained. The compound was recrystallized from methanol-ether; m.p., 179°.

$C_{15}H_{17}O_4N_3$ (279.3). Calculated, N 15.1; found, N 15.1

Carbobenzoxy- β -alaninamide—11.2 gm. of carbobenzoxy- β -alanine in 300 cc. of absolute methanol were esterified with dry HCl in the usual manner. The partly crystalline carbobenzoxy- β -alanine methyl ester was amidated in methanol-ammonia. On concentration *in vacuo*, 4.3 gm. of the compound were obtained. It was recrystallized from ethanol as elongated plates; m.p., 164°.

$C_{11}H_{14}O_2N_2$.	Calculated.	C 59.4, H 6.4, N 12.6
222.2	Found.	" 59.4, " 6.5, " 12.5

This compound was made from the acid chloride and NH_3 by Rapport *et al.* (21), who report a melting point of 163–164°.

β -Alaninamide Acetate—Hydrogenation of 2.5 gm. of the carbobenzoxy compound gave 1.4 gm. of prisms on standing with ether. After recrystallization from ethanol, the compound melted at 118°.

$C_6H_{12}O_2N_2$ (148.2). Calculated, N 18.9; found, N 19.1

The corresponding hydrochloride (m.p., 149°) has been described (22).

L-Alaninamide Hydrochloride—The syrupy ethyl ester hydrochloride from 15 gm. of L-alanine was amidated in the usual manner. The slight precipitate was discarded and the mother liquor concentrated *in vacuo*. Yield, 14.7 gm. The compound was obtained as fine needles after recrystallization from methanol-ether; m.p., 196–199°.

$C_3H_7ON_2Cl$ (124.6). Calculated, N 22.6; found, N 22.4

The corresponding DL compound (m.p., 170°) has been described (22).

Benzoyl-L-alaninamide—An aqueous solution of 3.1 gm. of L-alaninamide hydrochloride at 0° was treated with 10 gm. of sodium bicarbonate and 7 gm. of benzoyl chloride. Yield, 4.5 gm. of prisms; m.p., 235–240°, after recrystallization from hot water. The DL compound melts at 229–230° (22).

$C_{10}H_{12}O_2N_2$ (192.2). Calculated, N 14.57; found, N 14.57

SUMMARY

1. Crystalline pancreatic carboxypeptidase hydrolyzes carbobenzoxy-glycylamino acids about 800 to 1600 times faster than it does the corresponding carbobenzoxy- β -alanyl amino acids.

2. Leucine aminopeptidase of hog intestinal mucosa hydrolyzes L-leucyl- β -alanine at almost the same rate as L-leucylglycine, showing the aminopeptidase character of this enzyme. The crude aminopeptidase preparations of human uterus and rat muscle also hydrolyze L-leucyl- β -alanine. Further evidence is given that the uterine extract contains an enzyme, in addition to the aminopeptidase, which hydrolyzes L-leucylglycine.

3. The prolidases of hog intestinal mucosa and of rabbit muscle hydrolyze glycyl-L-proline about 200 to 300 times more rapidly than β -alanyl-L-proline. Moreover, the hydrolysis of glycyl-L-proline is inhibited about 50 per cent in the presence of an equimolar concentration of the β -alanyl compound.

4. The hydrolysis of glycyl-L-leucine by an enzyme from human uterus is about 200 times as fast as that of β -alanyl-L-leucine.

5. No hydrolysis of β -alanyl- β -alanine or of β -alanylglycine and only slight hydrolysis of glycyl- β -alanine were detected with preparations of human uterus or of rat muscle which are rich in glycylglycine dipeptidase.

6. An extract of rat muscle hydrolyzed triglycine, glycyl- β -alanylglycine, and diglycyl- β -alanine. The action on β -alanylglycylglycine was very slow and was lost on standing under conditions which did not influence the splitting of the other tripeptides.

7. The synthesis of a number of derivatives and peptides of β -alanine and of L-alanine is described.

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A RAPID METHOD FOR THE DETERMINATION OF CITRIC ACID*

By MURRAY SAFFRAN AND ORVILLE F. DENSTEDT

(From the Department of Biochemistry, McGill University, Montreal, Canada)

(Received for publication, June 2, 1948)

Numerous methods, enzymatic and chemical, have been reported for the determination of small amounts of citric acid in biological fluids. Of the chemical methods the majority involve the conversion of the citric acid to pentabromoacetone, which may be estimated by various gravimetric, titrimetric, or colorimetric procedures. The more recent modifications (1-3) of the method have simplified it and increased the sensitivity. Even in the simplified methods, however, the procedures are laborious. The method to be described is an application of the Furth-Herrmann reaction (4) and is considerably more rapid and simple to carry out.

Furth-Herrmann Reaction—The Furth-Herrmann reaction consists simply in taking up the deproteinized sample in warm acetic anhydride and adding pyridine. Citric acid gives a carmine-red color, aconitic acid violet-red, and tartaric acid emerald-green. The reaction must be carried out under anhydrous conditions. Gronvall (5), in 1937, used it in a roughly quantitative manner for estimating citric acid in the fluids of the eye, the water being removed by evaporation. Although the reaction gives satisfactory results with simple solutions of citric acid, the color development is erratic when the reaction is applied to dried residues of deproteinized serum. This difficulty may be overcome by using an excess of acetic anhydride for dehydration instead of evaporating the sample to dryness. Under these conditions both citric and aconitic acids, on the addition of pyridine, give a yellow color which is nearly proportional to the concentration. By careful control of conditions the modified method can be used to estimate the total of citric and aconitic acids, or aconitic acid alone. Citric acid may be obtained by difference. In practice, however, since the amount of aconitic acid in animal tissues and fluids is negligible, the method may be used for the direct estimation of citric acid.

* This work was incidental to a larger study on citric acid metabolism which was initiated under a grant from the Baxter Laboratories of Canada Limited, Acton, Ontario, and was continued during the tenure of a Life Insurance Medical Research Student Fellowship by one of us (M. S.). A preliminary communication was presented at the meeting of the Chemical Institute of Canada, Toronto, June 25, 1946.

Method

1 ml. portions of the deproteinized samples, containing 5 per cent trichloroacetic acid and between 15 and 400 γ of citric acid, are pipetted into dry colorimeter tubes. Exactly 8 ml. of *anhydrous* reagent grade acetic anhydride are then added from a burette, and the tubes are closed with rubber stoppers and are placed for 10 minutes in a constant level water bath kept at $60^{\circ} \pm 1^{\circ}$. Then exactly 1 ml. of *dry* reagent grade pyridine is added to each tube, and the tubes are restoppered as quickly as possible and are placed in the bath for 40 minutes. At the end of the period they are transferred to an ice-water bath for 5 minutes. Finally, they are wiped dry and the color is read in the Evelyn colorimeter, with Filter 420 or 400,

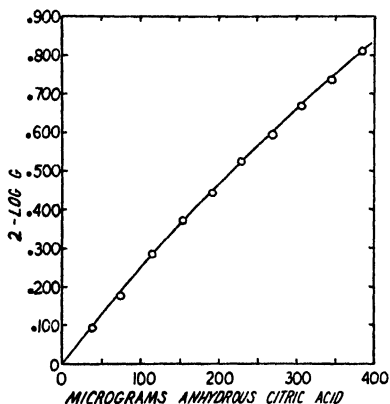


FIG. 1.

FIG. 1. Typical standard curve.

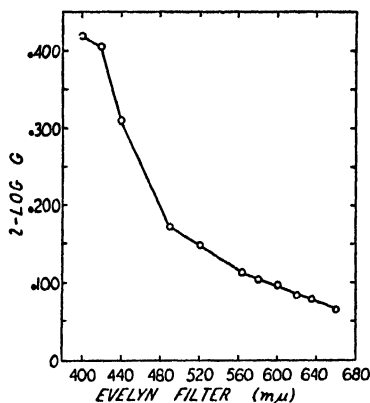


FIG. 2.

FIG. 2. Absorption of the color formed with citric acid measured by the Evelyn photoelectric colorimeter with various filters.

with the 10 ml. aperture. A blank, starting with 1 ml. of 5 per cent trichloroacetic acid solution, is carried through the same operations as described and is used to set the colorimeter.

Standard Curve—As the intensity of the color deviates slightly from Beer's law, it is desirable to include in each run one or two tubes with known amounts of citric acid. A standard curve is constructed with 1 ml. portions of citric acid solutions, containing from 15 to 400 γ , in 5 per cent trichloroacetic acid. A typical standard curve is shown in Fig. 1.

Choice of Filter—The light transmission with the colored solution and various Evelyn filters is illustrated in Fig. 2. Filter 400 gives only slightly greater sensitivity than does Filter 420. Absorption tests in the ultraviolet with the Beckman quartz spectrophotometer indicate an even greater sensitivity at still shorter wave-lengths.

Effect of Temperature—The intensity and stability of the color depend on the temperature. Acetic anhydride and the water of the sample react during a 10 minute heating at 60° to form acetic acid. When pyridine is added, heat is evolved so rapidly that the contents of the tube will boil if the temperature is not controlled. Although heat accelerates the development of the color, too high a temperature will cause it to fade rapidly after it reaches the maximum. At moderate temperatures the color is stable. Fig. 3 illustrates the effect of temperature on the development and intensity of the color. While heating at 40° yields the most intense color, a maximum is reached only in 2 hours. We have chosen 60° because of the more rapid development and excellent stability of the color.

Interfering Substances—Many organic acids, when used in relatively large amounts, yield a yellow color with acetic anhydride and pyridine.

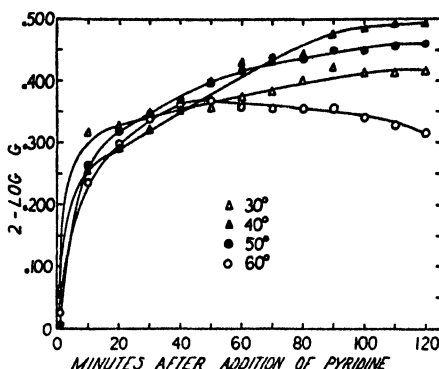


FIG. 3. Color development with citric acid at various temperatures. A Lumetron colorimeter, with the purple filter, was used to measure the color.

The small amounts of these substances present in animal tissues and fluids, with the exception of urine, however, cause no interference. Urine contains a chromogen which apparently is not citric acid, since removal of the latter by treatment with acid permanganate and potassium bromide, and extraction with petroleum ether, does not diminish greatly the yellow color formed with acetic anhydride and pyridine.

No considerable interference in the color reaction is caused by glutathione, glucose, urea, purified plasma protein, or by succinic, ascorbic, oxalic, and malonic acids. Fumaric, pyruvic, and *l*-malic acids give less than 1 per cent of the color produced by an equal amount of citric acid. Tartaric, itaconic and isocitric acids yield appreciable colors, but of these only isocitric acid occurs in the animal body.

Precision of Method—Satisfactory recoveries of added citric acid have been obtained from tissue extracts and suspensions (Table I), but recoveries

from serum are consistently low as 75 to 80 per cent of the theoretical values (Table II). Nevertheless, the method can be used satisfactorily with

TABLE I
Recoveries of Citric Acid from Fluid in Manometric Experiments

Tissue preparation	Citrate			Citrate recovered
	In sample	Added	Found	
	<i>micromole</i>	<i>micromoles</i>	<i>micromoles</i>	<i>per cent</i>
Rat kidney slices	0.00	1.00	1.06	106
	0.02	1.00	0.99	97
Pigeon liver homogenate	0.84	2.00	2.96	106
	0.86	2.00	2.96	105
	0.90	2.00	3.20	115
	0.94	2.00	3.08	107
Rabbit kidney homogenates	0.15	1.00	1.07	92
	0.15	1.00	1.10	95
	0.23	1.00	1.15	92
	0.24	1.00	1.15	91
	0.04	1.00	0.99	95
	0.03	1.00	1.04	101
	0.04	1.00	1.00	96
	0.03	1.00	1.02	99
	0.33	0.50	0.82	98
	0.31	0.50	0.79	96
	0.31	0.50	0.78	94
	0.32	0.50	0.79	94
	0.33	0.50	0.83	100

Average recovery, 98.9%; mean deviation, 5.3%

TABLE II
Recovery of Citric Acid from Serum

Kind of serum	Citric acid			Per cent recovered
	In sample	Added	Found	
	<i>micromole</i>	<i>micromoles</i>	<i>micromoles</i>	
Human, lyophilized and reconstituted	0.04	1.00	0.84	80
	0.04	1.00	0.79	75
	0.04	1.00	0.79	75
	0.00	1.00	0.77	77
Rabbit	0.84	1.05	1.64	76
	0.86	1.05	1.68	77

blood plasma or serum by multiplying the values by a factor of 1.3. It has been used in our studies on the rate of clearance of injected citric acid from the circulation.

Concentration of Trichloroacetic Acid—The low recoveries with serum seem to be related to the binding of trichloroacetic acid by the precipitated proteins, since the absorption of the blank is affected by the concentration of trichloroacetic acid (Fig. 4). This is suggested further by the improved recoveries obtained when the serum is diluted with Ringer's solution prior to the precipitation of the proteins (Fig. 5). As the dilution of the serum was increased, the recovery improved, and samples containing from 0 to 60 per cent serum in Ringer's solution yielded nearly 100 per cent recovery. The same results were obtained with 5 and 10 per cent trichloroacetic acid.

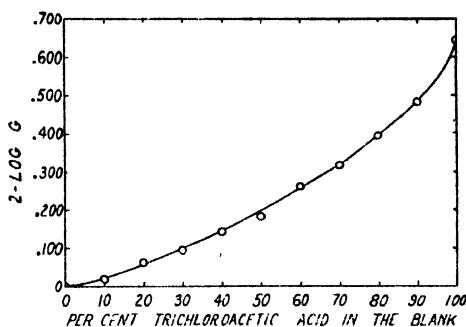


FIG. 4. Absorption at 420 $m\mu$ of the blank containing various concentrations of trichloroacetic acid. The colorimeter was set with the first tube, i.e. the tube containing no trichloroacetic acid.

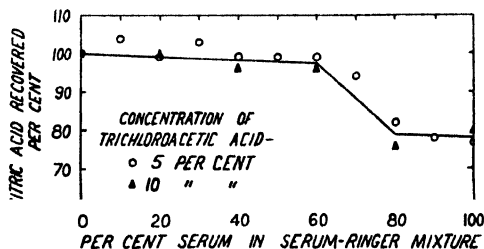


Fig. 5. Recovery of citric acid from serum-Ringer mixtures

It may be that the trichloroacetic acid takes part in the formation of the color and that the interference is due to the partial removal of trichloroacetic acid by precipitation along with the proteins.

For general application of the method it is important to adhere to the following conditions: (a) always use trichloroacetic acid for the removal of proteins; (b) keep the concentration of trichloroacetic acid in the filtrate at 5 per cent; and (c) use exactly 1 ml. of the filtrate for the estimation. This amount of filtrate should contain between 15 and 400 γ of citric acid. For example, with rabbit serum, which normally contains from 50 to 100 γ of citric acid per ml., one should add 1 ml. of 10 per cent trichloroacetic acid

to 1 ml. of serum. 1 ml. of the resulting filtrate (5 per cent trichloroacetic acid) then will contain 25 to 50 γ of citric acid per ml.

Determination of Aconitic Acid

By controlling the temperature the method can be used for the estimation of aconitic acid, since the latter compound yields a yellow color with acetic anhydride and pyridine at 0°, at which temperature citric acid does not

TABLE III
Optical Densities of Colors Formed by Tricarboxylic Acids at 0° and at 60°

Tricarboxylic acid	40 min. at 60°	60 min. at 0°
Citric.....	0.469	0.006
	0.465	0.006
<i>trans</i> -Aconitic.....	0.459	0.478
	0.472	0.469
<i>cis</i> -Aconitic.....	0.435	0.278
	0.426	0.276
Isocitric.....	0.116	0.006

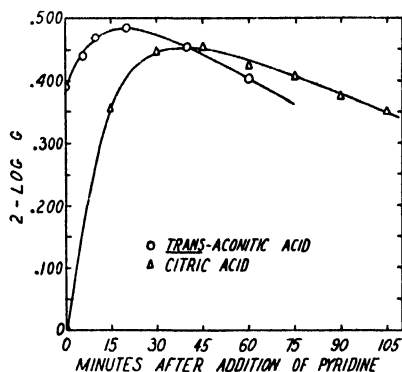


FIG. 6. Development of the color with citric acid and *trans*-aconitic acids at 60°

react. Table III indicates the behavior of several tricarboxylic acids at 0° and 60°. Only the *cis*- and *trans*-aconitic acids give colors within 60 minutes at 0°, but heating at 60° for 40 minutes yields the same color intensity with citric and *trans*-aconitic acids. *cis*-Aconitic acid yields about 90 per cent of the color intensity and isocitric acid only 25 per cent. Fig. 6 illustrates the development of the color with equivalent amounts of citric and *trans*-aconitic acids at 60°. It will be observed that the curves intersect (at 40 minutes), thus making possible the simultaneous estimation of these two compounds.

Method

The samples are prepared in the same way as for the determination of citric acid. 1 ml. of the sample, in 5 per cent trichloroacetic acid, is heated for 10 minutes in a stoppered colorimeter tube with 8 ml. of acetic anhydride at 60°, and the resulting solution is cooled in ice for 5 minutes. 1 ml. of cold pyridine then is added, the contents are mixed by shaking gently, and the tube is restoppered and replaced into the ice bath. After 60 minutes the tubes are wiped dry and read immediately in the colorimeter against an appropriate blank.

We wish to acknowledge the assistance of Miss Ann Puxley in verifying certain data presented in this paper.

A sample of the dimethyl ester of isocitric acid lactone was obtained through the courtesy of the late Dr. G. W. Pucher.

SUMMARY

A rapid method is described for the determination of small amounts of citric acid, based on the formation of a yellow color in the presence of trichloroacetic acid, acetic anhydride, and pyridine. By regulating the temperature this procedure can be used for determining aconitic acid, since only the unsaturated tricarboxylic acid yields the color at 0°.

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PURIFIED LIVER EXTRACT; CHEMICAL NATURE AS DETERMINED BY PAPER PARTITION CHROMATOGRAPHY

By GARSON H. TISHKOFF, ALEJANDRO ZAFFARONI, AND HENRY TESLUK

(From the Departments of Pathology and Biochemistry, School of Medicine and Dentistry,
The University of Rochester, Rochester, New York)

(Received for publication, March 16, 1948)

The biologically active constituents of liver extracts have been the subject of a considerable number of investigations aimed at elucidating the nature of the unidentified hematopoietic factor or factors. SubbaRow *et al.* (1) have reviewed in detail the studies concerned with the classical antipernicious anemia principle present in so called "purified" liver extracts.

This report presents the results of an investigation of purified liver extract containing a high concentration of the antipernicious anemia factor by the relatively new technique of two dimensional paper partition chromatography.^{1,2} The results indicate the presence of one or more polypeptides of high molecular weight, and this material has been separated from the free amino acids of the extract by filter paper partition technique. The free amino acids in the extract were identified as well as those liberated from the polypeptide material by hydrolysis. No appreciable quantities of free folic acid or xanthopterin could be detected.

The application of the paper partition method to peptide material has been demonstrated by several investigators. Thus Consden, Gordon, Martin, and Synge (2) have studied the partial hydrolysis products of gramicidin S, and Dent (3) has examined the peptides and amino acids which occur in biological material such as blood and urine. Dent, Stepka, and Steward (4) have extended the method to the peptides and amino acids of potato plant extracts, and Mider³ has conducted similar studies on animal tissue extracts. Goldberg, Gilda, and Tishkoff (5) have employed the paper technique in the analysis of salivary nitrogenous constituents.

Methods

The analytical method employed in this work was essentially that of Consden, Gordon, and Martin (6) as modified by Dent (3). The two

¹ We are grateful to Eli Lilly and Company for a supply of purified liver extract (15 U. S. P. units) and to Dr. J. M. Smith, Jr., of the American Cyanamid Company for a gift of xanthopterin and 2-amino-4-hydroxypteridine-6-carboxylic acid.

² We wish to acknowledge the many suggestions and technical supervision of Dr. C. E. Dent of the University College Hospital, London.

³ Mider, G. B., unpublished work.

solvents consisted of highly purified phenol and of a mixture of collidine (2,4,6-trimethylpyridine) and lutidine (2,4-dimethylpyridine). The individual amino acids were identified by their relative positions on the paper square and identity was confirmed by comparison with a reference chromatogram.⁴ The relative positions were found to be more reliable than the absolute R_F values. An R_F value is a fraction equivalent to the distance a substance has been moved by the solvent divided by the distance of the leading edge of the solvent. An estimation of the amino acid concentrations was made by assigning to the spots numbers ranging from 1 to 10 according to an arbitrary scale of values of intensity. A value of 1 was given to the spot in which the color was just perceptible (about 1 γ of amino acid) and a value of 10 to those showing an intense purple color. Proline and hydroxyproline were not rated, since they give a yellow color with ninhydrin. By comparison of the color intensity as well as the spot size with hydrolysates of pure proteins, an approximate estimate could be made (± 30 per cent). All hydrolyses were carried out in sealed tubes at 100° with either 6 N HCl or 7 N NH_4OH . The excess acid or alkali was removed by repeated evaporations *in vacuo*, and the residue was then redissolved in sufficient water to give the original volume.

EXPERIMENTAL

Fig. 1 shows a diagram of a chromatogram made with 10 microliters of the commercial extract without previous hydrolysis. The presence of a considerable quantity of free amino acids was definitely established. No unidentifiable spot was observed. Leucine (not distinguishable from isoleucine and norleucine), phenylalanine, valine, and tyrosine were the principal free amino acids found. Other amino acids were barely detectable or entirely absent. It was observed, however, that the absolute R_F values for the amino acids in phenol were appreciably less than the reported values, although the values in collidine-lutidine were normal. This anomaly was attributed to the presence of a high molecular weight compound in the extract and will be discussed in detail below.

When the paper chromatogram was held in front of a strong ultraviolet light source, an intense yellow-green *fluorescent spot* was observed in a location to the right of the phenylalanine spot. This observation was always made before development of the chromatogram with ninhydrin. In order to establish the identity of this substance, a number of one dimensional chromatograms were made of several fluorescent compounds known to be present in liver. Of the compounds studied, riboflavin was the only one which had the same R_F values in phenol and collidine-lutidine

⁴ We are indebted to Dr. C. E. Dent for an excellent reference diagram, prior to publication, of many naturally occurring amino compounds.

as the fluorescent compound present in the liver extract. This was confirmed by the addition of riboflavin to a casein hydrolysate as well as to the liver extract; in the latter case, only one fluorescent spot was observed.

Folic acid was also considered as a possible constituent of the extract. Although pure folic acid itself fails to show any appreciable fluorescence, it does give a well defined yellow colored spot owing to its intrinsic color. Moreover when folic acid is subjected to alkaline hydrolysis, a characteristic fluorescent spot appears on the chromatogram with an R_f value in

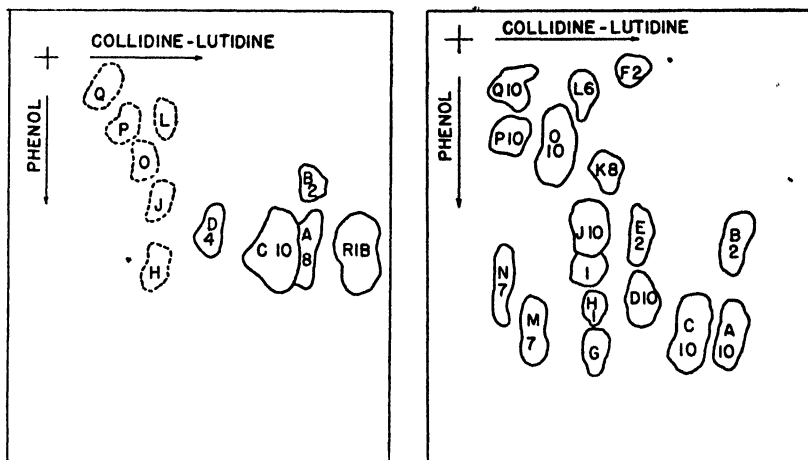


FIG. 1. Diagrams of paper chromatograms made with liver extract. Left, 10 microliters of liver extract; right, 5 microliters of hydrolyzed liver extract. The sample was placed at the upper left corner. The dotted lines indicate weak spots. The numbers represent relative color strength. Cystine (or cysteine) appears after conversion to cysteic acid by hydrogen peroxide. A phenylalanine, B tyrosine, C leucine, D valine, E methionine sulfone, F cysteic acid, G proline, H histidine, I hydroxyproline, J alanine, K threonine, L serine, M arginine, N lysine, O glycine, P glutamic acid, Q aspartic acid, RIB riboflavin.

phenol of 0.43 and in collidine-lutidine of 0.24. This fluorescent compound has the same R_f values as a pure sample of 2-amino-4-hydroxypteridine-6-carboxylic acid.¹ Since no such spot was observed with liver extract hydrolyzed by alkali, we have concluded that folic acid (or its conjugate) was not present in the original extract in any appreciable amounts. These results agree with those of several other investigators who also could not detect folic acid in liver extract (7, 8).

It was found that *xanthopterin* yields a characteristic fluorescent spot on the chromatogram with an R_f value in phenol of 0.67 and in collidine-lutidine of 0.34. Since no such spot was obtained from the unhydrolyzed

liver extract, we have also concluded that xanthopterin is not present in the original extract.

When liver extract is subjected to acid hydrolysis, a large increase was observed in the intensities of the spots on the ninhydrin-developed chromatogram owing to the amino acids mentioned above (with the exception of tyrosine), and, in addition, spots appeared, owing to large relative quantities of glycine, glutamic acid, aspartic acid, serine, threonine, alanine, arginine, lysine, proline, and hydroxyproline. Methionine and cysteine were likewise shown to be present by converting them respectively to methionine sulfone and cysteic acid by treatment of the hydrolysate with 30 per cent hydrogen peroxide. Tryptophan is completely destroyed by acid hydrolysis and hence is not observed on the chromatogram of the acid hydrolysate. However, after alkaline hydrolysis a tryptophan spot was observed. A chromatogram made with 5 microliters of the undiluted acid-hydrolyzed extract is shown in Fig. 1. All amino acids had normal R_f values in both solvents. Since a large number of amino acids appeared after hydrolysis, one or more substances of polypeptide nature must have been present in the original extract. The presence of at least one compound (presumably polypeptide) of reasonably high molecular weight is also indicated by the observed retardation of the free amino acids in the phenol direction when a chromatogram was made with the unhydrolyzed extract, as previously mentioned. *The retardation of amino acids* by high molecular weight constituents such as proteins and large *polypeptides* has been commonly observed by Dent in this laboratory as well as during our own studies. It may be pointed out that after hydrolysis of the extract no retardation effects were observed. This retardation occurs only in the first solvent used, since the amino acids, once freed of the interfering substance by use of the first solvent, will move normally in the second solvent. The peptides which are present do not give a positive ninhydrin test, since they do not appear on the developed chromatogram; hence, only a small proportion of free amino groups can be present in the molecules. Nevertheless, lysine, which is a diamino acid, appeared in the chromatogram after hydrolysis.

Fractionation of Liver Extract

Our attention became directed toward the possibility of isolating such a polypeptide substance. For this purpose, a fractionation of the unhydrolyzed liver extract was accomplished by paper chromatography by the method of Dent (3). Twenty samples of 10 microliters each of extract were placed near one edge of the paper square. After running the paper in the collidine-lutidine mixture, a strip was cut from the lateral edge and developed with ninhydrin as a single one dimensional chromatogram.

With this strip as a reference guide, the remainder of the sheet was divided arbitrarily into eight strips parallel to the edge on which the samples were originally placed. Thus every strip contained nineteen samples and had an approximate R_F range of 0.1. The strips were then placed in a water trough and the individual fractions were washed off, evaporated to dryness, hydrolyzed, and diluted to 190 microliters. A two dimensional chromatogram of each of the hydrolysates of the fractions was then made. Since identity of the free amino acids present in the fraction of each R_F range before hydrolysis was known, a good approximation of the amino acid composition of the peptide substance present in the fraction of the same R_F range could be obtained.

Of the fractions studied, only Fractions 5 through 7 showed any appreciable amino acids liberated on hydrolysis. It follows that the peptide

TABLE I
Relative Concentration of Amino Acids Present in Fraction 5
 R_F value 0.25 to 0.35.

High	Medium	Low
Leucine (10)	Arginine (4)	Histidine (1)
Glycine (10)	Lysine (4)	Cysteine (2)
Alanine (10)	Glutamic (6)	Methionine (2)
Aspartic (10)	Serine (6)	
Valine (8)	Phenylalanine (3)	
Proline	Threonine (3)	
	Hydroxyproline	

The numbers following the amino acid indicate the relative amount of the amino acid in the peptide.

substance possessed an absolute R_F range in the collidine-lutidine mixture of 0.25 to 0.60. Since every fraction showed the same relative amounts of amino acids liberated as closely as could be estimated, it is probable that only a single polypeptide was present, although further studies are needed to verify this. Table I summarizes the amino acids liberated in Fraction 5, which was considered to be the fraction least contaminated with free amino acids and probably most indicative of the polypeptide composition.

DISCUSSION

Previous attempts to identify the antipernicious anemia principle have associated the activity of extracts with a high molecular weight polypeptide (1). Furthermore the free amino acids and riboflavin which occur in the extract are known to be inactive. It is also of interest that of the amino acids given in Table I many are found among the principal amino acids

reported in the best preparation of the active principle by Dakin and West (1). In particular, glycine, leucine, aspartic acid, proline, hydroxyproline, and arginine were noted. It is highly probable that we have been concerned with one and the same peptide. The possible similarity between this peptide substance and the polypeptide growth factor in liver described by Woolley (strepogenin) remains to be ascertained.

SUMMARY

Studies by paper chromatography of a highly active commercial preparation of the antipernicious anemia factor have shown (1) the presence of a material (or materials) of peptide nature, (2) the presence of some free amino acids, and (3) the presence of riboflavin. Folic acid and xanthopterin were not found in the extract. By use of the paper chromatography technique, the material (or materials) of peptide nature, which presumably is the active constituent, was separated from the free amino acids, and the amino acids obtained by hydrolysis of the peptide material were then identified by the paper chromatography technique.

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THIOURACIL AND THE CONVERSION OF CAROTENE TO VITAMIN A IN THE RAT*

By BARBARA KELLEY AND HARRY G. DAY

(From the Department of Chemistry, Indiana University, Bloomington)

(Received for publication, June 7, 1948)

Several observations have indicated that the conversion of carotene to vitamin A is impaired by a hypothyroid state (1). However, the results of recent studies on the problem are not in agreement (2-6). Determinations of vitamin A in the tissues have been made in only two of the investigations (5, 6). According to Wiese *et al.* (5), thiouracil does not affect the conversion of carotene to vitamin A as indicated by the level of vitamin A in the liver following the administration of carotene to thiouracil-fed rats on a vitamin A-low diet. Johnson and Baumann (6) found that less vitamin A is formed from carotene when the thyroid activity is impaired with thiouracil in the drinking water or with thiourea in the food.

We were investigating this problem when the recent papers appeared. Because our results seemed to confirm the findings of Johnson and Baumann (6), that aspect of the study was discontinued. However, the summarized findings are reported here owing to the importance of the problem and the lack of agreement in the literature.

EXPERIMENTAL

Young rats were given a vitamin A-deficient diet composed of vitamin A-free casein 18, glucose 58, salts 4 (7), brewers' yeast 8, irradiated yeast 2, and hydrogenated cottonseed oil (Crisco) 10. After 25 to 30 days the diet was altered to contain 0.5 per cent of 2-thiouracil. The animals were continued on this regimen until growth was impaired and a small percentage had xerophthalmia. At this time they were divided as equally as possible according to litter, weight, sex, and incidence of xerophthalmia. One group in each series remained on the diet containing thiouracil. The other group in each series was given the same diet except that the thiouracil was replaced by 0.6 per cent thyroid "globulin,"¹ a thyroid concentrate containing 0.79 per cent iodine. It was determined by preliminary experiments that the thyroid preparation was capable of slightly decreasing the growth rate of rats when fed at a level of 0.6 per cent. Also, the thiouracil was sufficient

* Aided by a grant from Standard Brands Incorporated, and by the Research Funds of the Graduate School of Indiana University.

¹ Furnished by The Maltine Company, through the courtesy of Dr. R. L. Kroc.

to produce hyperplasia of the thyroid. Subsequently both groups in each series were given β -carotene (90 per cent β - and 10 per cent α -carotene) in wheat germ oil,² as a single dose in two series and in divided doses in two other series. 12 hours after the last dose of carotene the animals were sacrificed. The livers and, in one series, the kidneys were analyzed for vitamin A.

The tissue samples were homogenized with a Waring blender with approximately 5 ml. of water per gm. of tissue. The subsequent saponification of the tissues is facilitated by this treatment. The homogenates were saponified at 50° with an equal volume of 15 per cent alcoholic potas-

TABLE I

Effect of Feeding Thiouracil and Thyroid "Globulin" on Content of Vitamin A in Liver of Vitamin A-Depleted Rats following Supplementation with β -Carotene

Series No.	No. of rats	Days of thiouracil feeding	Days of thyroid feeding	Total carotene fed	Length of time on carotene supplements	Vitamin A			
						Total	P		P
				γ	hrs.	γ		γ per gm.	
I	5	33	0	2,000	12	107 $\pm$ 6.9*		14.0 $\pm$ 1.9*	
	3	25	8	2,000	12	170 $\pm$ 17.6	0.045	21.3 $\pm$ 3.6	0.06
II	5	40	0	3,000	12	249 $\pm$ 10.6		71.4 $\pm$ 4.7	
	5	31	9	3,000	12	360 $\pm$ 24.9	<0.01	93.0 $\pm$ 5.8	0.04
III	7	29	0	350	84	75 $\pm$ 4.4		14.0 $\pm$ 1.5	
	7	23	6	350	84	85 $\pm$ 7.3	0.30	16.1 $\pm$ 1.7	0.40
IV	3	40	0	10,000	972	165 $\pm$ 12.5		30.8 $\pm$ 2.8	
	3	0	40	10,000	972	155 $\pm$ 20.6	>0.5	20.9 $\pm$ 5.1	0.32

* The standard error of the mean. P was calculated by the method of Snedecor (9).

sium hydroxide. The saponified mixtures were extracted with five 50 ml. portions of freshly distilled diethyl ether. The extracts were washed with water and dried over anhydrous potassium carbonate. The water-free samples were then evaporated to dryness and the residues taken up in chloroform. Vitamin A was determined on the chloroform solutions by the colorimetric method of Sobel and Werbin (8).

The principal results are summarized in Table I. In animals given a single dose of carotene (Series I and II) the total amount of vitamin A in the liver, as well as the concentration, was definitely lower in those given the thiouracil without the thyroid preparation. In Series I, given 2000 γ of carotene, the probability was only 0.045 that chance factors could

* Furnished by the VioBin Corporation, through the courtesy of Mr. Ezra Levin.

account for the difference in total vitamin A. Thus the difference must be regarded as significant. Series II, given 3000 γ of carotene, showed an even greater difference between rats continued on the thiouracil and those changed to the thyroid substance 9 days before administering the carotene. In this case, as would be expected, the concentration of the vitamin, as well as the total amount, was higher than in Series I. With regard to the total amount of vitamin A, the probability was less than 0.01 that the difference was fortuitous. There was a probability of only 0.040 that chance accounted for the difference in concentration. The livers of the thiouracil-fed rats had 71.4 ± 4.7 γ of vitamin A per gm. and those given the thyroid substance had 93.0 ± 5.8 γ per gm.

Such large differences were not found in the livers of rats given only 350 γ of carotene over a period of 3 days (Series III). The livers of those receiving thiouracil had 14.0 ± 1.5 γ per gm., whereas those fed the thyroid substance had 16.1 ± 1.7 γ per gm. Although this indicates that the former animals were able to utilize less of the carotene, the difference may not be significant, as shown by a probability value of 0.40. In addition, Series IV, given 10,000 γ of carotene per rat over a period of 40 days, appeared to show as much vitamin A in the livers of those given thiouracil as in those fed the thyroid substance. In this series the carotene supplements were started at the same time the thiouracil and thyroid preparation were added to the diet.

The kidneys of the rats of Series III were analyzed for vitamin A. Although the results are not recorded in Table I, it suffices to state that the thiouracil-fed animals had 4.2 ± 0.8 γ per gm. as compared with 6.1 ± 1.7 γ per gm. for those given the thyroid concentrate. This apparent difference, although not statistically significant ($P = 0.36$), is of the same magnitude as that found in the liver vitamin A of these animals.

DISCUSSION

Because the experimental procedures used in this investigation differed considerably from those of Johnson and Baumann (6), it is particularly significant that the results from the two studies are in substantial agreement. Thus, in spite of contrary evidence based on the results from eight rats given thiouracil and six rats which served as controls (5), the conclusion seems to be warranted that the thyroid does affect the conversion of carotene to vitamin A.

Since thiouracil feeding has been found to retard the rate of depletion of the hepatic stores of vitamin A (10), it is necessary to recognize that the amount of vitamin A found in the tissues may be the resultant of two opposite effects of the thiouracil: (a) the impairment of carotene conversion to vitamin A and (b) the retention of vitamin A in the liver once it is deposited

there. On this basis the thiouracil-fed animals analyzed a short time after receiving a carotene supplement would be expected to have less vitamin A in the tissues than controls given a thyroid preparation. On the other hand, animals given thiouracil for many days after beginning the carotene supplements might be expected to retain more of the vitamin A formed from the carotene. The amount might be great enough to equal or possibly to exceed the difference due to the impairment in the conversion of carotene to vitamin A. This interpretation appears to be in agreement with the data presented here.

SUMMARY

2-Thiouracil impairs the conversion of carotene to vitamin A, as indicated by the content of vitamin A in the livers of vitamin A-deficient rats given β -carotene.

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α -AMINO- β , β -DIMETHYL- γ -HYDROXYBUTYRIC ACID; A PRECURSOR OF PANTOIC ACID*

BY W. W. ACKERMANN AND WILLIAM SHIVE

(From the Biochemical Institute and the Department of Chemistry, The University of Texas, and the Clayton Foundation for Research, Austin)

(Received for publication, March 22, 1948)

Ivanovics (1, 2) demonstrated that the toxicity of salicylic acid for *Escherichia coli* was concerned with pantothenic acid, probably in the production of pantoic acid or its conversion to pantothenic acid. Protein hydrolysates or mixtures of amino acids were effective in preventing the toxicity of the inhibitor. Under proper conditions, valine was found to exert a pronounced effect; however, other amino acids were also effective in preventing the toxicity. Kuhn and Wieland (3) reported the synthesis of pantoic acid from the keto acid corresponding to valine and suggested that the biosynthesis may proceed by a similar mechanism through α -keto- β , β -dimethyl- γ -hydroxybutyric acid which was reduced by yeast to pantoic acid.

In the present investigation, DL- α -amino- β , β -dimethyl- γ -hydroxybutyric acid, for which we suggest the name *pantonine* in order to stress its relationship to pantoic acid, was synthesized and found to be just as effective as pantoic acid in preventing the toxic action of salicylic acid for *Escherichia coli*. However, it was inactive in replacing pantoic acid in promoting the growth of *Acetobacter suboxydans*, which requires pantothenic acid or pantoic acid for growth. These and related experiments described below indicate the possibility that α -amino- β , β -dimethyl- γ -hydroxybutyric acid may be a precursor of pantoic acid.

EXPERIMENTAL

DL- α -Amino- β , β -dimethyl- γ -hydroxybutyric Acid (DL-*Pantonine*)— α , α -Dimethyl- β -hydroxypropionaldehyde (10 gm.), prepared from isobutyraldehyde and 40 per cent formaldehyde solution (4), was dissolved in 15 cc. of a cold saturated solution of ammonia in methanol. After 30 minutes, 6.5 gm. of sodium cyanide and 8 gm. of ammonium chloride in 36 cc. of water were added to the methanol solution. The reaction mixture was allowed to stand 18 hours at room temperature. The mixture was then extracted with four successive 50 cc. portions of ether. The ether extracts were combined, evaporated to 100 cc., and extracted with dilute hydro-

* From part of a thesis submitted by W. W. Ackermann to The University of Texas in partial fulfillment of the requirements of the degree of Doctor of Philosophy.

chloric acid. The acid extract was refluxed several hours and evaporated to dryness *in vacuo*. The residue was twice treated with acetone and filtered. The combined filtrates were evaporated to dryness under reduced pressure, and chloride ion was removed by the successive use of lead oxide and silver carbonate in aqueous suspension. The filtrate was treated with hydrogen sulfide, filtered, and evaporated to dryness to obtain 1 gm. of the free acid, melting at 244° after recrystallization from aqueous alcohol.

Analysis— $C_8H_{11}O_3N$. Calculated, N 9.52; found, N 9.54

Testing Methods—The method of testing with *Escherichia coli* has been previously described (5). The medium was modified by the omission of casein digested with trypsin, and the organism was carried on the salts-glucose medium without the aspartic acid supplement.

The medium used in tests with *Acetobacter suboxydans* has been described by Sarett and Cheldelin (6) as a medium for pantoic acid assay.

Results

The relative effect of DL- α -amino- β,β -dimethyl- γ -hydroxybutyric acid and DL-valine in preventing the toxicity of salicylic acid is shown in Table I. In the presence of 500 γ per 10 cc. of DL-valine, approximately 10 times as much salicylic acid is required for complete inhibition of growth as in its absence. The effect of DL- α -amino- β,β -dimethyl- γ -hydroxybutyric acid at the same concentration as valine was even more pronounced, in that the salicylic acid concentration had to be increased approximately 100-fold in order to inhibit growth completely.

As indicated in Table II, the relative activities of DL- α -amino- β,β -dimethyl- γ -hydroxybutyric acid and DL-pantoic acid in preventing the toxicity of 2 mg. of salicylic acid for *Escherichia coli* are approximately the same. In separate experiments, α -amino- β,β -dimethyl- γ -hydroxybutyric acid and pantoic acid at various concentrations were found to be practically identical in their ability to prevent the toxic action of increasing concentrations of salicylic acid. Of the known naturally occurring amino acids, leucine, isoleucine, and valine were the most effective in preventing the toxicity of salicylic acid; however, these amino acids, singly or as a mixture, did not prevent the toxicity of relatively high concentrations of salicylic acid which were prevented by pantoic acid or α -amino- β,β -dimethyl- γ -hydroxybutyric acid.

DL- α -Amino- β,β -dimethyl- γ -hydroxybutyric acid was found to be inactive in replacing the growth-promoting properties of pantoic acid or pantothenic acid for *Acetobacter suboxydans*. At concentrations up to 100 γ per 10 cc. of DL- α -amino- β,β -dimethyl- γ -hydroxybutyric acid, there was no response above the blank readings (7.0) obtained when the organism was

incubated for 3 days at 30° in the absence of pantothenic acid or pantoic acid. By contrast, a galvanometer reading of 78.0 was obtained when 0.5 γ of DL-pantoic acid per 10 cc. was present.

TABLE I

Relative Effect of α -Amino- β , β -dimethyl- γ -hydroxybutyric Acid and Valine on Toxicity of Salicylic Acid

Test organism, *Escherichia coli*, incubated 16 hours at 37°.

Salicylic acid γ per 10 cc.	Galvanometer readings*		
	Reversing agent, 500 γ per 10 cc.		
	None	DL- α -Amino- β , β -dimethyl- γ -hydroxybutyric acid	DL-Valine
0	52.8	52.0	52.2
30	52.3	52.5	52.2
100	3.3	49.2	49.0
300	2.0	48.2	48.5
1,000		45.5	5.0
3,000		31.0	2.0
10,000		3.0	

* A measure of culture turbidity; distilled water reads 0, an opaque object 100

TABLE II

Relative Effect of Pantoic Acid and α -Amino- β , β -dimethyl- γ -hydroxybutyric Acid on Toxicity of Salicylic Acid

Test organism, *Escherichia coli*, incubated 16 hours at 37°.

Reversing agent*	Galvanometer readings	
	DL-Pantoic acid	DL- α -Amino- β , β -dimethyl- γ -hydroxybutyric acid
γ per 10 cc.		
0	2.0	2.0
50	3.0	4.5
100	10.5	10.0
200	18.8	19.5
400	36.0	36.5
800	44.0	47.0
1000	46.8	49.0

* In the presence of 2 mg. of salicylic acid per 10 cc.

Conversion of α -Amino- β , β -dimethyl- γ -hydroxybutyric Acid to Pantoic Acid—In separate experiments, DL- α -amino- β , β -dimethyl- γ -hydroxybutyric acid was treated with nitrous acid at pH 4. The excess nitrous acid

was destroyed, and the reaction mixture assayed for pantoic acid with *Acetobacter suboxydans*. The product derived from 0.5 γ of the amino acid added to 10 cc. of medium was sufficient to give almost maximum response by the organism, indicating that a good conversion of the amino acid to pantoic acid was obtained. This served as a possible basis for an assay for DL- α -amino- β , β -dimethyl- γ -hydroxybutyric acid in natural extracts. However, only slight activity was obtained when a number of natural extracts were treated in this manner. The possibility that one of the optically active forms of α -amino- β , β -dimethyl- γ -hydroxybutyric acid on treatment with nitrous acid may give rise only to the biologically inactive form of pantoic acid requires further investigation before the presence or absence of pantonine in natural extracts can be determined by this method. Further investigation of the possible natural occurrence of this amino acid is in progress both with the above method and with paper chromatography.

DISCUSSION

Since α -amino- β , β -dimethyl- γ -hydroxybutyric acid (pantonine) was as effective as pantoic acid in preventing the toxic action of salicylic acid for *Escherichia coli*, the conversion of this amino acid to pantoic acid appears to take place in this organism which synthesizes pantoic acid from glucose as a source of carbon. On the other hand, *Acetobacter suboxydans*, which cannot synthesize pantoic acid from any known source of carbon, also cannot utilize α -amino- β , β -dimethyl- γ -hydroxybutyric acid in place of pantoic acid. These results suggest the possibility that α -amino- β , β -dimethyl- γ -hydroxybutyric acid may be a normal precursor of pantoic acid.

SUMMARY

DL- α -Amino- β , β -dimethyl- γ hydroxybutyric acid (pantonine) has been synthesized and found to be as effective as DL-pantoic acid in preventing the toxicity of salicylic acid for *Escherichia coli*, but was inactive in replacing the pantoic acid or pantothenic acid requirement of *Acetobacter suboxydans*. These results suggest the possibility that this amino acid may be the normal precursor of pantoic acid in the biological synthesis of pantothenic acid.

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ON THE ABSORPTION OF PHOSPHOLIPIDES*

BY CAMILLO ARTOM AND MARJORIE A. SWANSON

(From the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest College, Winston-Salem, North Carolina)

(Received for publication, March 4, 1948)

It is generally assumed that phospholipides are hydrolyzed in the gastrointestinal tract and absorbed in the form of their constituent parts. Indeed, enzymes capable of splitting phospholipides have been found in many tissues, including intestinal mucosa, pancreas, and pancreatic juice. The earlier findings have been reviewed by Belfanti, Contardi, and Ercoli (2), and additional evidence on this point was reported subsequently (3-5). Very recently Le Breton and Pantaléon (6) have conclusively shown that the pancreatic juice of the dog liberates both fatty acid radicals from mixtures of lecithins and cephalins, and that the enzyme responsible for this action is probably different from the lipase which hydrolyzes neutral fats.

On the other hand, several authors have found more or less significant increases in the lipide phosphorus of the lymph (Slowtyoff (7), Eckstein (8)) or blood (Eichholtz (9)) during the absorption of a large dose of lecithin. Since similar changes have been described after feeding neutral fat and since a possible rôle of phospholipides as an intermediary stage in the absorption of fatty acids has been postulated, the interpretation of these findings is rather uncertain. However, after prolonged feeding of phospholipides, increases have been found in the phospholipide content of various tissues (Rewald (10), Serijski (11), Heinlein (12)). The increases do not occur when the bile is excluded from the intestine, in which condition an impairment in the absorption of phospholipides can be demonstrated (Heinlein (12)). It seems, therefore, that one cannot dismiss the possibility of a direct absorption of intact phospholipides, a possibility which is also suggested by the ease with which these compounds are emulsified in water.¹

We thought that the use of radioactive phosphorus as a tracer offered a good opportunity to investigate this question. Labeled phospholipides

* Aided by a grant from the John and Mary R. Markle Foundation. The radioactive phosphorus used in the experiments was supplied by the Clinton Laboratories, Oak Ridge, Tennessee, on allocation from the United States Atomic Energy Commission. A preliminary report was presented before the annual meeting of the American Society of Biological Chemists, Atlantic City, March, 1948 (1).

¹ The problem may have also a certain practical interest, since, as pointed out by Bloor (13), phospholipides constitute about 3 per cent of hen's eggs and the use of phospholipides from soy beans and other sources for the preparation of commercial food products is markedly increasing.

were fed to rats and a study was made of the distribution of the isotope between the lipides and other phosphorus-containing fractions in the plasma and liver. The liver was included because it is believed that the synthesis of plasma phospholipide occurs mainly in the liver (14-16), and also because it has been shown that, when isotopic phospholipides in the form of fine emulsions (17, 15) or contained in the plasma of a donor (18-20) are introduced in the circulation, a large proportion is taken up by the liver. In the present investigation the results of the experiments in which labeled lipides were administered have been compared with those of control experiments in which the isotope was fed as inorganic phosphate or as glycerophosphate.

EXPERIMENTAL

Preparation of Materials Fed—The labeled phospholipides were obtained from the livers of animals (one rabbit or several rats) injected with large doses of P^{32} as Na_2HPO_4 . After 8 hours they were killed, the livers extracted with hot alcohol, the solvent removed under reduced pressure, and the residue taken up in chloroform. The chloroform solution was filtered through asbestos and concentrated to a small volume. The phospholipides were then precipitated with acetone and $MgCl_2$, and the precipitation was repeated twice. The precipitated phospholipides were further purified by a technique essentially similar to that described by MacLean (21), as adapted to animal tissues by Le Breton (22).² The emulsions fed to the rats were prepared by pouring the ether solution of the purified phospholipides over a very dilute aqueous solution of sodium glycocholate and evaporating the ether with a stream of air. Three lots of such emulsions (designated Emulsions L-1, L-2, and L-3) were employed in the various experiments. They had the following characteristics: Emulsion L-1 (rabbit liver), 131 mg. of phospholipides per cc., 403 counts per mg. of phosphorus; Emulsion L-2 (rat liver), 62 mg. of phospholipides per cc., 8800 counts per mg. of phosphorus; Emulsion L-3 (rat liver), 58 mg. of phospholipides per cc., 35,600 counts per mg. of phosphorus.³

In one control experiment (No. IIc), a portion of the same lot of labeled phospholipides from which Emulsion L-1 was prepared was hydrolyzed by boiling for 1 hour with alcoholic $Ba(OH)_2$. After addition of water and acidification with H_2SO_4 , the fatty acids were extracted with ether. From the aqueous layer, inorganic phosphate was removed by adding an excess of

² Even after three precipitations the acetone precipitate from liver lipides may still contain as much as 20 per cent of non-phospholipide phosphorus. These "impurities" seem to be effectively eliminated by the technique adopted, since in the purified liver phospholipide the molar ratio of phosphorus to fatty acid corresponds to the theoretical value in the lecithins and cephalins (Artom (23)).

³ The number of counts per minute as determined on our scale-of-eight impulse counter under the standard conditions of our measurements.

barium acetate and bringing to pH 8. The barium glycerophosphate was precipitated by 3 volumes of ethanol in the cold. This precipitate was redissolved in water, any insoluble material being rejected, and the barium removed by cautious addition of H_2SO_4 . The solution of labeled glycerophosphate was neutralized with NaOH and sodium glycocholate was added. The fatty acids which had been extracted from the hydrolyzed phospholipides were then emulsified with the solution of glycerophosphate and glycocholate as described above. This emulsion (LH-1) contained 2 mg. of phosphorus per cc. and gave 403 counts per mg. of phosphorus. Less than 20 per cent of the phosphorus was soluble in chloroform.

In the other control experiments, non-isotopic phospholipides were emulsified in aqueous solutions of sodium glycocholate and Na_2HPO_4 containing P^{32} . Two lots of these emulsions (C-2 and C-3) were prepared, having approximately the same phospholipide content and the same radioactivity as Emulsions L-2 and L-3, respectively. Because of the high specific activity of the phosphate in Emulsions C-2 and C-3, each animal received no more than 0.02 mg. of phosphorus as inorganic phosphate.

Absorption Experiments—White rats maintained on a stock diet (Rockland Farms) were fasted for 16 hours and then given by stomach tube 2 to 3 cc. of the various emulsions. The amounts actually ingested were estimated from the difference between the radioactivity in the emulsion fed and that found in the washings of the syringe and stomach tube. 3 or 6 hours after feeding, the rats were bled from the severed neck vessels. In order to increase the amount of blood which could be obtained, heparin was injected 45 minutes before bleeding. The gastrointestinal tract was washed with several portions of a dilute NaOH solution, alcohol, and ether, and the radioactivity was determined on an aliquot of the combined washings. The results were used to evaluate the extent of the absorption.

Analytical Procedures—In most of our experiments, two or three rats were used and the blood plasma pooled. The liver analyses were carried out individually, except in Experiments Ia, Ib, Ic, and IVb, in which pooled samples were used. The plasma and one liver sample were treated with cold 10 per cent trichloroacetic acid, and the precipitate washed twice with the same solution. The lipides were extracted from the residue with alcohol, alcohol-ether, and chloroform (24). Another sample of the liver was minced under alcohol and the lipides extracted directly without preliminary treatment with trichloroacetic acid.⁴ From the acid extracts of the plasma

⁴ In the two samples of the liver the figures for the specific activity were very similar. However, the lipides directly extracted from the liver with alcohol and alcohol-ether usually gave higher values for both phosphorus and radioactivity than those obtained on the sample which had been first treated with trichloroacetic acid. In neither sample was the radioactivity appreciably lowered by subsequent shaking with pulverized sodium phosphate. On the other hand, in control experiments in

and liver, aliquots were taken and treated as follows: (a) An aliquot was brought to pH 6 with NH_4OH , and the phosphate precipitated with strychnine molybdate reagent (25). The precipitate, washed with 1 per cent HNO_3 and with water, was dissolved in acetone and the solution labeled "inorganic phosphate." (b) Another aliquot was incinerated with H_2SO_4 and HNO_3 , the digest neutralized, and the phosphate precipitated with the strychnine molybdate reagent. The acetone solution of the precipitate was designated "total acid-soluble P." The difference between the values determined in fractions (b) and (a) was termed "organic acid-soluble P." (c) A third aliquot was brought to pH 8.2 with $\text{Ba}(\text{OH})_2$. The precipitate was discarded, and 3 volumes of alcohol added to the supernatant solution. After 24 hours in the refrigerator, the precipitate was taken up in water, any insoluble matter being rejected, and the solution labeled "glycerophosphate." Since the amounts of plasma available were insufficient, the glycerophosphate fraction was not isolated from the plasma extracts.⁵

In the lipide extracts and in the various fractions of the trichloroacetic acid extracts, the radioactivity and the phosphorus content were determined as previously described (15). The radioactivity data are expressed as "relative radioactive units" (r.r.u.), the amount of isotope absorbed by the animal being considered equal to 10^4 r.r.u. The ratio between the radioactivity (in r.r.u.) and the phosphorus content (in mg.) is designated "specific activity."

RESULTS AND DISCUSSION

Data on the experimental conditions, on the weights of the animals and livers, and on the absorption of the radioactive materials are recorded in

which both methods were applied to two samples of the same rat plasma, identical values were obtained. It may be pointed out that the weight of the dry materials in the liver samples (1.5 to 2.5 gm.) was much higher than in the sample of the plasma (200 to 300 mg.). It seems probable, therefore, that the preliminary treatment by trichloroacetic acid made the extraction of lipides more difficult, and that, after such treatment, the extraction was complete only when, as in the analysis on the plasma, the volumes of the solvents used were very large compared to the size of the sample.

⁵ It is realized that these fractions may contain phosphorus from components other than those named. The precipitation of inorganic phosphate was made at room temperature at pH 1 or less for 20 to 30 minutes. It is probable that a certain proportion of easily hydrolyzable phosphate compounds such as creatine phosphate or adenosine triphosphate may be split. However, since the amount of these compounds in the liver is small compared to that of the inorganic phosphate, the admixture of phosphorus from such a source presumably does not constitute a serious error. On the other hand, the significance of the "glycerophosphate" figures is more questionable, since one-fifth to one-fourth of the phosphorus in the alcohol precipitate may be due to Ba hexose monophosphate (Kaplan and Greenberg (26)).

Table I. When the isotope was given in the form of inorganic phosphate or glycerophosphate, the absorption was practically complete in 3 hours. On the other hand, after feeding radioactive phospholipides, the absorption of P^{32} was always lower and varied considerably from animal to animal, even in the same experiment. A large proportion had already been absorbed

TABLE I
Absorption of Labeled Phosphorus Compounds in Rats

Experiment No.	Rat No.	Final body weight	Liver weight	Emulsion fed	Compounds labeled	P^{32} absorbed	
						Counts per min.	Per cent
3 hr. experiments							
Ia	9	gm. 115	gm. 5.60	L-3	Phospholipides	177,400	70
	10	122	4.56	"	"	133,100	57
Ib	11	123	5.51	"	"	174,600	75
	12	117	4.52	"	"	142,000	63
Ic	18	150	6.83	C-3	Inorganic phosphate	202,000	97
	19	146	5.82	"	" "	199,400	95
	20	137	6.14	"	" "	202,100	97
6 hr. experiments							
IIa	1	105	4.46	L-1	Phospholipides	3,100	49
IIb	3	124	5.57	"	"	2,000	40
IIc	2	100	4.88	LH-1	Glycerophosphate	2,100	95
IIIa	6	101	3.38	L-2	Phospholipides	27,200	51
	7	93	2.63	"	"	43,100	70
	8	97	3.48	"	"	45,700	81
IIIb	4	145	5.93	C-2	Inorganic phosphate	25,000	99
	5	124	3.89	"	" "	25,200	98
IVa	13	112	4.44	L-3	Phospholipides	159,400	74
	14	130	4.72	"	"	175,300	73
IVb	15	150	6.83	C-3	Inorganic phosphate	202,000	98
	16	146	5.82	"	" "	199,400	97
	17	137	6.14	"	" "	202,100	96

after 3 hours and no consistent increase in this proportion in the longer time interval is apparent from the limited number of our data.⁶

The interpretation of our results is to a large extent dependent upon the relations between the specific activity values in the various fractions ana-

⁶ If one attempts to estimate from the absorption of P^{32} the amounts of phospholipides absorbed by a 125 gm. rat (a procedure which is obviously open to question), the average figures would be 115 mg. absorbed after 3 hours and 148 mg. after 6 hours.

lyzed. Accordingly, only these values are given in Figs. 1 and 2. The points represent the results of the individual experiments, each value being obtained from at least two animals (either determined individually or in duplicate on pooled samples). In the control animals which were fed inorganic phosphate, at both time intervals, the specific activities of the plasma and liver phospholipides are practically identical, and much lower than that of all the other fractions. These gradients are in agreement with the con-

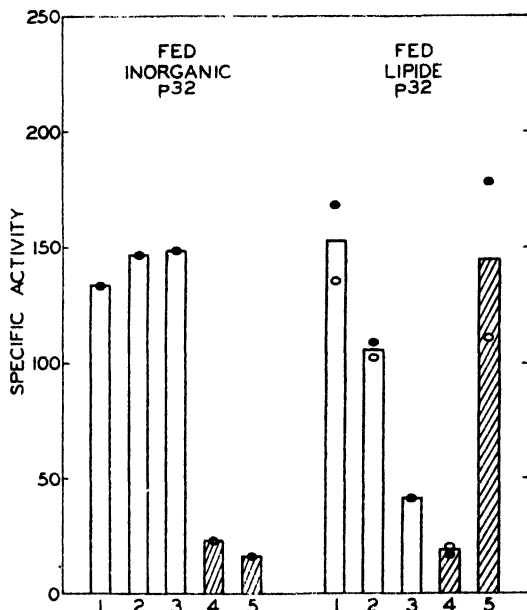


FIG. 1. Specific activities of P fractions in plasma and liver of rats 3 hours after feeding inorganic P³² or lipid P³². In each group the bars represent the following fractions: 1, plasma inorganic P; 2, liver inorganic P; 3, liver glycerophosphate P; 4, liver lipid P; 5, plasma lipid P. The points represent individual experiments (see Table I); rats fed inorganic P (●, Experiment Ic); lipid P (●, Experiment Ia; ○, Experiment Ib).

cept that the phospholipides synthesized in the liver from the inorganic phosphate are rapidly exchanged with those of the plasma (14-16). On the other hand, when the isotope was fed as phospholipide, the specific activity of the plasma lipides, at both time intervals, was much higher than that of the controls, and also much higher than that of the liver lipides in the same animal. Since it has been shown that the specific activity of a product cannot be higher than that of its precursor before the maximum specific activity of the precursor has been reached (27, 28), and since in our experiments the specific activity of the liver lipides in-

creases markedly between the 3rd and 6th hours, these facts together constitute definite evidence that the liver phospholipides could not have been the sole source of the plasma phospholipides. It appears, therefore, that a portion of the labeled phospholipides which were fed must have been absorbed as an intact molecule.⁷

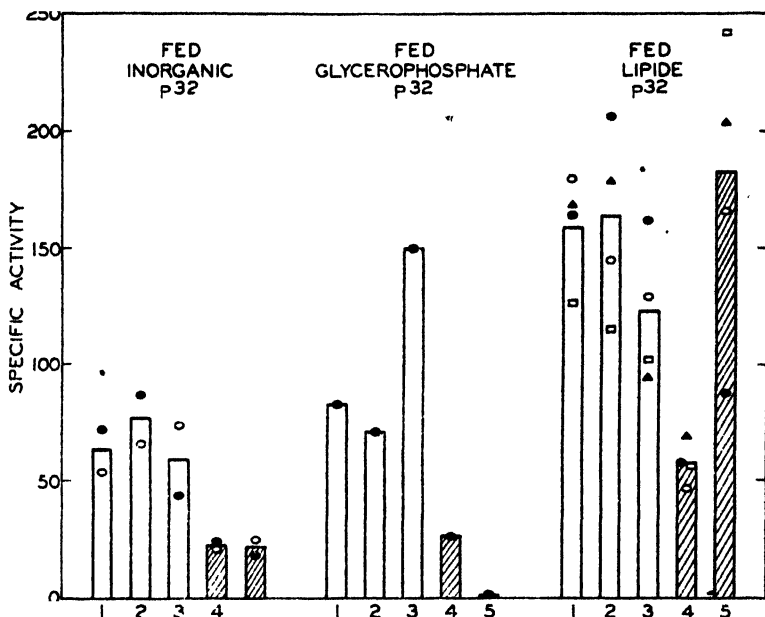


FIG. 2. Specific activities of P fractions in plasma and liver of rats 6 hours after feeding inorganic P³², glycerophosphate P³², or lipide P³². In each group the bars represent the following fractions: 1, plasma inorganic P; 2, liver inorganic P; 3, liver glycerophosphate P; 4, liver lipide P; 5, plasma lipide P. The points represent individual experiments (see Table I); rats fed inorganic P³² (●, Experiment IIb; ○, Experiment IVb); glycerophosphate P³² (●, Experiment IIc); lipide P³² (●, Experiment IIa; ○, Experiment IIb; □, Experiment IIIa; ▲, Experiment IVa).

The finding of large amounts of radioactivity in the inorganic phosphate as early as the 3rd hour is evidence for a fairly rapid splitting of the fed lipides. It seems probable that most of this splitting occurred in the intestinal tract, where enzymes capable of actively hydrolyzing phospholipides have been demonstrated (2-6). However, the possibility that some hy-

⁷ By "intact phospholipide molecule" we mean all the phosphorus-containing compounds which are insoluble in trichloroacetic acid but soluble in alcohol-ether and chloroform. Possible split-products which may be included are those from which one fatty acid or the nitrogenous component has been removed.

drolysis of absorbed phospholipides also occurred in various tissues cannot be excluded (see below, section (f)).

The possibility of a direct absorption of glycerophosphate was tested by feeding this substance containing P^{32} . In this experiment the specific activity of the glycerophosphate in the liver was markedly higher than that of the inorganic P. Similar results were not found in the animals which received labeled phospholipides. It seems likely that glycerophosphate liberated from the phospholipides in the gastrointestinal tract at a comparatively slow rate may be easily hydrolyzed to inorganic phosphate before absorption. However, when large amounts of the free ester are fed at one time, a notable proportion may be absorbed even before being split, thus becoming an important source of the glycerophosphate in the liver.

We believe that the results of our experiments under various conditions are qualitative evidence that the phosphorus present in the phospholipide molecule can be absorbed in more than one form. On the other hand, in the present state of our knowledge, it does not seem possible to calculate from our data the relative importance of each of these processes because of the large number of unknown variables.

Besides this evidence concerning the absorption of phospholipides, several accessory findings which may be of interest can be mentioned.

(a) In spite of the large differences in the radioactivity of the materials fed in the 6 hour experiments, the results are essentially similar. This similarity seems to exclude the possibility that the metabolic processes were detectably modified in 6 hours by the highest doses of P^{32} absorbed (as high as 21 microcuries). Also this similarity is indirect evidence for the reliability of our radioactive measurements even in certain cases (*e.g.* plasma lipides in Experiments IIa, IIb, and IIc) in which the actual counts were very low and therefore subject to a large statistical error.

(b) The levels of lipide P in the plasma of our rats varied between 1.6 and 2.5 mg. per 100 cc. These values are surprisingly low in comparison with those found in the plasma of other animal species. However, as mentioned before,⁴ we do not think that these low values can be ascribed to an incomplete extraction of lipides from the trichloroacetic acid precipitate of the plasma. Furthermore, figures of the same order of magnitude have been obtained in this and other (29) laboratories by the direct extraction of rat plasma with alcohol and alcohol-ether.

(c) The specific activity of the plasma inorganic phosphorus was higher in the animals fed labeled lipides than in those fed inorganic P^{32} , the differences being especially marked in the 6 hour experiments. These findings could be due to a continuing liberation of inorganic P from the lipides either in the gastrointestinal tract or in other tissues after absorption (see section (f) below). In individual rats differences were also found between the

specific activities of the inorganic phosphate in plasma and liver. Many of these differences were well beyond the limits of experimental error and suggest that the exchange of phosphate between plasma and liver requires a definite amount of time.

(d) Glycerophosphate may be one intermediary compound in the synthesis or breakdown of phospholipides. In our experiments the specific activity of the liver glycerophosphate was considerably higher than that of the liver lipides and (with the exception of the experiment in which labeled glycerophosphate was fed) generally lower than that of the inorganic phosphate. This gradient is not incompatible with a rôle of glycerophosphate in the synthesis of phospholipides. On the other hand, the data suggest that the chief *immediate* source of the phosphorus of the glycerophosphate is the inorganic rather than the lipide phosphorus. Since it has been shown that there is no physical exchange *in vitro* between phosphate and glycerophosphate (30), and since the specific activity of these fractions was often rather close to one another, it appears from our results (in agreement with those of others (26, 31)) that the *biological* turnover of glycerophosphate proceeds fairly rapidly.

(e) For the sake of brevity, figures for the specific activity of the organic acid-soluble P were not included in the drawings. In the liver, these values ranged from 40 to 80 per cent of those of the glycerophosphate isolated from the same fraction (and therefore they were considerably higher than the specific activity of the liver lipides). It is conceivable that the unknown immediate precursor of the phospholipides is included in the organic acid-soluble P but, since it may be only a minor component, it seems to us that deductions based on the specific activity of the whole mixture would have little significance.

(f) When the total amount of radioactivity which was recovered in all fractions of the plasma and liver is calculated, marked differences are found between the animals fed labeled inorganic P and those fed labeled phospholipides. 3 hours after feeding the marked sodium phosphate, 1051 r.r.u. were recovered; at the 6th hour, only an average of 605 r.r.u. Since the inorganic P^{32} is rapidly exchanged with the phosphate of the bones and also partially removed from the body by excretion, the decrease observed at the longer time interval is quite consistent with the data showing that practically all of the phosphate fed was absorbed in the first 3 hours. On the other hand, an average of 582 r.r.u. was found in the liver and plasma of rats killed 3 hours after feeding labeled phospholipides, whereas, at the 6th hour, 1062 r.r.u. were found. This increase could logically be ascribed to a continuing supply of isotope from the intestine, but such an interpretation does not agree with the finding that the amount of P^{32} absorbed between the 3rd and the 6th hours was apparently small. The hypothesis might perhaps be

suggested that most of the phospholipides which were absorbed in the early period were temporarily distributed among various tissues. From there they may be slowly transferred to the liver, as intact molecules or as hydrolytic products, thus accounting for the maintenance of high specific activity values in the inorganic and lipide P fractions of the plasma. The marked increase in the specific activity of the liver lipides between the 3rd and 6th hours (in contrast to the almost unchanged values in the control experiments) may be accounted for partially by synthesis from the inorganic phosphate of increasing specific activity and partially by exchange with plasma phospholipides of very high specific activity.

SUMMARY

Labeled phospholipides (prepared from the liver of animals injected with radioactive phosphate) were fed by stomach tube to rats. After 3 or 6 hours the radioactivity and the phosphorus content were determined in various fractions of the plasma and liver. The results were compared with those obtained on control rats receiving non-labeled phospholipides and P^{32} as sodium phosphate (or glycerophosphate).

From such a comparison, it appears that phospholipides may be absorbed at various stages of hydrolysis. While part of the phospholipides is split in the gastrointestinal tract, so that the phosphate radical is absorbed in inorganic form (some possibly as glycerophosphate), there is also definite evidence that a detectable portion of the ingested phospholipides can be absorbed as the intact molecule.

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CRITIQUE OF METHODS FOR THE DETERMINATION OF RIBOFLAVIN IN URINE*

By ELMER DE RITTER, MARY E. MOORE, ERICH HIRSCHBERG, AND SAUL H. RUBIN

(From the Nutrition Laboratories, Hoffmann-La Roche, Inc., Nutley, New Jersey)

(Received for publication, May 29, 1948)

Both fluorometric and microbiological measurements of urinary riboflavin excretion have served as a basis for evaluation of nutritional status, detection of deficiency states, measurement of physiological availability, and determination of daily requirements. Fluorometric assay methods have commonly followed three basic procedures; namely, (a) that of Najjar (1), based on the extraction of riboflavin into a butanol-pyridine medium; (b) the double hydrosulfite reduction method of Hodson and Norris (2); and (c) the Florisil method of Ferrebee (3). Most microbiological assays have been based on the *Lactobacillus casei* assay of Snell and Strong (4).

However, the literature shows that considerable disagreement exists in regard to the reliability and relative advantages of the commonly used methods. For example, in a series of availability tests, Melnick *et al.* (5) report Strong and Carpenter's (6) modification of the Snell and Strong method to be more specific for urinary riboflavin than the method of Najjar with the blank correction omitted, but these authors prefer the fluorometric method because of the better reproducibility. Keys *et al.* (7) compared the microbiological method of Snell and Strong with a modification of the Conner and Straub (8) Florisil procedure and found the latter considerably more satisfactory for urinary riboflavin assay. Recently, Slater and Morell (9) have developed a modification of the Najjar procedure and found it to show agreement for urine assay with an unpublished microbiological method with *Lactobacillus casei*. In a comparative study of fluorometric methods, Morell and Slater (10) found many methods to be non-specific for urinary riboflavin. These included a Florisil method utilizing the adsorption-elution technique of Barton-Wright and Booth (11), the direct and indirect methods of Najjar (1), and Hodson and Norris (2) procedure, the direct method of Ferrebee (3) with an added internal standard, and a method involving oxidation with cold KMnO_4 and H_2O_2 , followed by a hydrosulfite blank.

* Publication No. 140.

Presented before the American Institute of Nutrition at the Thirty-second annual meeting of the Federation of American Societies for Experimental Biology at Atlantic City, March, 1948.

In the course of determinations in this laboratory of the physiological availability of riboflavin from enriched rice (12) and from soluble derivatives of riboflavin (13),¹ according to the technique of Melnick *et al.* (5), comparisons have been made of several fluorometric procedures with modifications of the Snell and Strong microbiological assay. The fluorometric methods included the modified Hodson-Norris procedure which we have described for low potency foods (14), several modifications of the Conner and Straub Florisil method, and the procedure of Slater and Morell. In attempts to evaluate the specificity of the various methods, particularly with regard to possible compounds of riboflavin, the effect of clarase digestion on microbiological and Florisil assays and the effect on the former of sterilization by aseptic filtration as compared to autoclaving were investigated. The rôle of urea as an inhibitor of titrimetric microbiological assays was also studied. The urines in these availability determinations contained normal or elevated amounts of riboflavin. A recent study in this laboratory of the urinary excretion of B vitamins during intravenous feeding (15) and a number of urines from children on low riboflavin diets (16) afforded the opportunity to extend these comparisons to a very low range of riboflavin concentration.

EXPERIMENTAL

Assay Methods—Microbiological riboflavin assays were carried out by the method of Snell and Strong (4), based on the growth of *Lactobacillus casei*; for reasons outlined below, turbidimetric measurement of growth after 24 hours, with an Evelyn photoelectric colorimeter with a 660 m μ filter, was routinely preferred to measurement by titration after 72 hours.

In fluorometric assays by the direct method of Rubin *et al.* (14), an aliquot of acidified urine of about 1 per cent of the 24 hour volume (to the nearest 5 ml.) was treated with 1.0 ml. of 4 per cent KMnO₄, followed after 2 minutes with sufficient 3 per cent H₂O₂ to decolorize or precipitate the excess permanganate. After adjustment of the pH to 6 with sodium acetate, dilution to a concentration of about 0.1 γ of riboflavin per ml., and filtration, the usual double reduction procedure was followed.

The Florisil method of Conner and Straub (8) was modified as follows. Before fluorometry, 15 ml. aliquots of the pyridine-acetic acid eluates were treated with 1 ml. of 4 per cent KMnO₄ for 1 minute and the excess KMnO₄ decolorized with just sufficient 3 per cent H₂O₂; internal standards were read after pipetting 0.2 ml. (1.5 γ) of the standard riboflavin solution into the cuvette containing 15 ml. of the KMnO₄-treated eluates. The procedure of Slater and Morell (9) was carried out as described by these authors.

¹ Rubin, S. H., Haas, G. J., Drecker, L., De Ritter, E., and Hirschberg, E., unpublished data.

Photochemical destruction of riboflavin was avoided by working as much as possible in a dark room illuminated by Wratten safelights, series 0A, and by the use of low actinic glassware elsewhere.

Effect of Urea on Microbiological Assays—Since the usual microbiological procedure involves sterilization of the assay tubes by autoclaving, it was suspected that, in the case of urine, hydrolysis of urea to NH_3 during autoclaving might lower the amount of alkali needed to titrate the lactic acid formed and thus lead to erroneously low assay values by the titrimetric method. In view of various conflicting reports on the effect of urea reported in the literature (17–20), it was decided to test this point as follows. Urea was added to pure riboflavin solutions in the ratios of 10, 25, and 45 gm. per mg. of riboflavin to correspond to low, medium, and high levels of

TABLE I
Effect of Urea on Microbiological Riboflavin Assays

Urea per mg. of riboflavin	Relative response of <i>Lactobacillus casei</i>			
	Turbidimetric		Titrimetric	
	Autoclaved	Filtered aseptically	Autoclaved	Filtered aseptically
gm.				
0	1.00	1.00	1.00	1.00
10	1.01	1.04	0.99	1.02
25	1.00	1.02	0.97	1.02
45	0.99	0.99	0.92	1.01

urea excretion. Sterilization by autoclaving was compared with sterilization by filtration through glass; growth of *Lactobacillus casei* was measured by titration after 72 hours of incubation as well as turbidimetrically after 24 hours. Table I shows the results obtained.

These data indicate that urea does not interfere with turbidimetric or glass-filtered titrimetric assays, but at the higher levels of urea a slight lowering of titrimetric values occurs after autoclaving. Although on diets of average protein content the excretion of urea is too small to lower the titrimetric riboflavin values significantly, the 24 hour turbidimetric measurement was chosen for routine use because of its speed and convenience as well as its complete independence of the effect of urea. In low riboflavin urines collected from patients on a regimen of total intravenous feeding of protein hydrolysates (*cf.* Table V), the estimated proportion of urea to riboflavin may be as high as 200 gm. of urea per mg. of the vitamin, so that the choice of the turbidimetric method becomes obligatory. For example, for a urine containing approximately 150 gm. of urea per mg. of riboflavin,

the titrimetric assay was only 0.05 mg. per day as compared to 0.20 mg. by turbidimetric measurement and 0.24 mg. by the Florisil method.

Comparison of Microbiological and Double Reduction Methods—Since Morell and Slater (10) have reported the original Hodson and Norris

TABLE II
Comparative Assays of Basal and Test Urines (Microbiological versus Direct Fluorometric)

Subject	No. of samples	Average riboflavin content		Mean difference,* $\frac{Fl - M}{M} \times 100$	Test of significance	
		Direct fluoro-metric assay	Microbiological assay		t found	t limit at 5 per cent level
Basal urines						
E. D. R.	9	mg. per day 1.51	mg. per day 1.43	per cent 5	2.5	2.3
L. D.	9	0.69	0.52	44	2.5	2.3
F. W. J.	6	0.89	0.75	21	5.6	2.6
S. H. R.	8	1.22	1.00	22	5.9	2.4
E. S.	8	1.96	1.78	10	4.9	2.4
J. C. B.	4	1.29	1.18	12	2.1	3.2
J. S.	7	0.98	0.87	11	2.9	2.4
E. H.	5	1.72	1.64	10	0.9	2.8
Average.	7	1.28	1.15	17		
Test urines						
E. D. R.	9	6.5	6.3	3	0.8	2.3
L. D.	9	5.6	5.5	4	0.5	2.3
F. W. J.	6	4.9	5.3	-8	2.5	2.6
S. H. R.	8	6.8	6.5	7	1.7	2.4
E. S.	8	7.9	7.7	4	1.0	2.4
J. C. B.	4	6.1	6.5	-7	2.6	3.2
J. S.	7	6.2	6.1	1	0.1	2.4
E. H.	5	6.9	6.7	3	0.6	2.8
Average.....	7	6.4	6.3	1		

* The mean difference was obtained by calculating the per cent difference for each urine, then averaging these differences for each subject.

method (2) to measure appreciable amounts of non-riboflavin fluorescence, our modification of this method was investigated to determine its specificity for urinary riboflavin. Comparative assays by the latter procedure and by the microbiological method are given in Table II. These basal and test urines were collected during availability studies from normal males on adequate but different diets. Consistently higher values were obtained on

basal urines by the double reduction method, the average differences ranging from 0.08 to 0.22 mg. per day or from 5 to 44 per cent. In all but two cases, these differences are statistically significant. For the high potency test urines, both methods are in good agreement, none of the differences being significant.

Comparison of Fluorometric and Microbiological Methods—A comparison of the Florisil, Slater and Morell, double reduction, and microbiological assays of normal urines is given in Table III. The Florisil values are intermediate in all cases between the higher values by the double reduction method and the lower values by the Slater and Morell procedure. The microbiological values fall between the Slater and Morell and the Florisil values. Although the better agreement of these three methods indicates

TABLE III

Comparison of Fluorometric and Microbiological Urinary Riboflavin Assays
All values in mg. per 24 hours.

Subject	Fluorometric assay			Microbiological assay*
	Double reduction method (Rubin <i>et al.</i>)	Florisil method (Conner and Straub)	Extraction into non-aqueous medium (Slater and Morell)	<i>Lactobacillus casei</i> (Snell and Strong)
E. S.	0.99	0.88	0.72	0.87
F. W. J.	0.84	0.65	0.54	0.56
L. D.	0.68	0.43	0.30	0.34
S. H. R.	3.64	3.45	3.22	3.28
E. D. R.	1.32	1.19	1.09	1.19

* No significant differences were observed after sterilization by autoclaving or aseptic filtration.

greater specificity as compared to the double reduction method, the possibility was considered that the latter procedure, in which the fluorescence of the treated urine solution is measured directly, may measure some combined form of riboflavin which the other methods do not measure. We (13) have previously shown that microbiological assays of riboflavin derivatives are not necessarily as high as fluorometric or biological assays. Moreover, Rosner, Lerner, and Cannon (21) have demonstrated that combined forms of riboflavin are not adsorbed by Florisil as readily as free riboflavin but can apparently be converted to free or adsorbable riboflavin by enzyme digestion. To test this point, the effect of enzyme digestion on urinary riboflavin was studied microbiologically and by the Florisil technique of Rosner *et al.* (21). Aliquots of urine containing approximately 50 γ of riboflavin were adjusted to pH 4.5 and digested with 0.25 gm. of clarase for

3 hours at 45°. Assays before and after enzyme treatment are given in Table IV. In several cases the severe washing of the Florisil, which Rosner *et al.* have shown to remove combined riboflavin, produced slightly lower values than the usual washing with 45 ml. of hot water, but the values after

TABLE IV

Effect of Clarase Digestion on Florisil and Microbiological Assays*

All values in mg. per 24 hours.

Subject	Florisil assay				Microbiological assay	
	Untreated		Clarase-treated		Untreated	Clarase-treated
	Usual wash (45 ml.)	Severe wash (150 ml.)	Usual wash (45 ml.)	Severe wash (150 ml.)		
F. W. J.	0.53	0.45	0.58	0.48	0.59	0.61
E. D. R.	1.36	1.29	1.33	1.33	1.38	1.36
E. S.	1.60	1.53	1.68	1.57	1.64	1.75
S. H. R.	0.83	0.82	0.81	0.84	0.91	0.91
L. D.	0.41	0.40	0.44	0.38	0.46	0.48

* Approximately 50 γ of riboflavin + 0.25 gm. of clarase digested 3 hours at 45° at pH 4.5.

TABLE V

Comparative Assays of Low Riboflavin Urines

All values in mg. per 24 hours.

Subject	Sample No.	Fluorometric assay		Microbiological assay <i>Lactobacillus casei</i> (Snell and Strong)
		Double reduction method (Rubin <i>et al.</i>)	Florisil method (Conner and Straub)	
T. J.	1	0.88	0.79	0.73
	2	0.60	0.45	0.42
	3	0.27	0.11	0.12
	4	0.30	0.24	0.20
	5	0.38	0.30	0.27
G. T.	1	0.89	0.70	0.57
	2	0.28	0.13	0.10
	3	0.31	0.13	0.10
	4	0.45	0.19	0.19
	5	0.26	0.13	0.11

enzyme digestion were not significantly higher with either the washing or microbiological procedure. Hence, there is no evidence of combined riboflavin.

Comparative Assays of Low Riboflavin Urines—Table V shows the results of comparative assays of urines collected from two subjects who were

kept on total intravenous feeding with protein hydrolysate (15). With no measurable intake of riboflavin, the excretion of this vitamin rapidly reaches a very low level. Again, the double reduction values are higher than the Florisil and microbiological assays, which are in relatively good agreement.

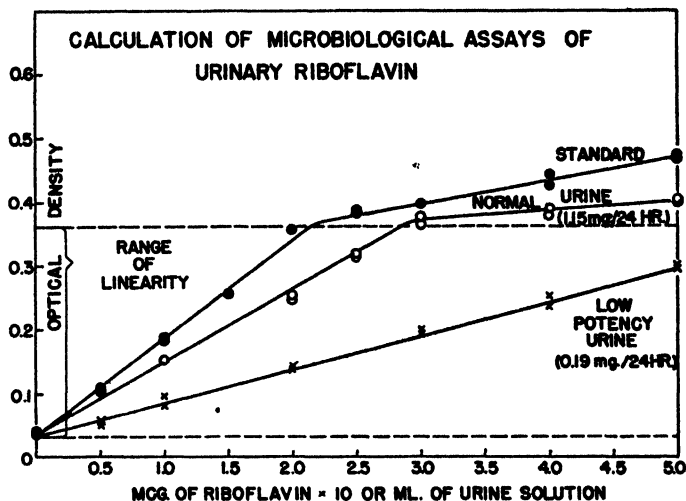


FIG. 1

TABLE VI
*Comparative Assays of Low Riboflavin Urines**

All values in micrograms per 24 hours.

Sample No.	Fluorometric assay			Microbiological assay
	Double reduction	Florisil	Slater and Morell	
K1	59	62	33	33
K2	75	80	45	43
Z1	60	72	44	39
Z2	61	84	53	50

* Supplied through the courtesy of Dr. L. E. Holt, Jr., and Dr. S. E. Snyderman, Department of Pediatrics, New York University College of Medicine, and the Children's Medical Service of Bellevue Hospital, New York.

Evidence of the validity of the microbiological assays, even for these low potency urines, is given in Fig. 1, which represents a typical plot of such an assay for calculation by the slope-ratio method of Wood (22, 23). The fact that both standard and test points follow straight lines in the same response range, which have practically the same intercept on the vertical axis of response, indicates that the assays are valid.

A similar comparison, including also the Slater and Morell method (9), has been made (*cf.* Table VI) for extremely low riboflavin urines collected from children on experimental diets in the course of studies on minimum human requirements by Snyderman *et al.* (16). The method of Slater and Morell yielded consistent agreement with microbiological assays, whereas both the Florisil and double reduction values were considerably higher.

DISCUSSION

The possibility that urea, in the amounts present in urine, might lower the microbiological assays was investigated because of the conflicting data reported previously. Feeney and Strong (17) and Strong *et al.* (18) experienced no difficulty in the determination with or without the addition of urine from which riboflavin had been removed by photolysis or adsorption on Lloyd's reagent. On the other hand, Fraser *et al.* (19) stated that the urine of some riboflavin-depleted dogs showed the presence of one or more substances inhibitory for the growth of *Lactobacillus casei*. Isbell *et al.* (20) showed that this inhibition was due to the presence of urea. The present studies show a slight lowering of titrimetric values after autoclaving urines containing high levels of urea, but the effect is somewhat smaller than that found by Isbell *et al.* (20) at comparable ratios of urea and riboflavin. Since no effect on turbidimetric assays was found, even at ratios 5 times larger than that found when the diet is high in protein, the turbidimetric method is preferable in this respect for urinary riboflavin assays.

Among the fluorometric methods tested, that of Slater and Morell (9) is the only one to show agreement with microbiological values at extremely low as well as at normal riboflavin levels. Our modification (14) of the double reduction procedure gives consistently higher values, but the amount of non-riboflavin fluorescence measured is less than that found by Morell and Slater (10) with the original Hodson and Norris procedure (2). This difference is apparently due to the use of permanganate oxidation and of a minimum amount of hydrosulfite for blank determinations in the present modification. Application of the Florisil technique of Rosner *et al.* (21) before and after clarase digestion failed to show the presence of combined riboflavin which could lead to higher values by the double reduction method than by Florisil or microbiological assays.

Values obtained for normal urines by the Florisil procedure show better agreement with microbiological assays than that reported by Morell and Slater (10), who measured considerable "apparent riboflavin" in the Florisil eluates. The use of boiling water to wash the Florisil columns and of permanganate oxidation of eluates in the present method may be responsible for elimination of much non-riboflavin fluorescence, although Morell and

Slater report the formation of "apparent riboflavin" during such oxidation of urine itself. Although difficulties with the present lot of Florisil have been encountered in previous assays of a variety of products (14, 24), it is apparent that the Florisil and the technique used were reliable for assay of normal urines. Possible variations in the performance of different batches of Florisil may lead to difficulties in various laboratories similar to those encountered in the assay of cereal products (25). For extremely low potency urines, the values obtained by riboflavin assays by both the Florisil and double reduction methods are considerably higher on a percentage basis than the values by the microbiological or Slater and Morell method. The absolute differences are relatively small, possibly because of the highly purified nature of the children's diet.

When specificity is of primary importance, the Slater and Morell method, which includes a test for specificity by successive, controlled exposures to sunlight, is the most reliable of the fluorometric methods tested, particularly at very low riboflavin levels. It is more time-consuming than the Florisil and double reduction procedures because of the fact that a complete recovery test is run with each sample. When complete specificity is not required, as in physiological availability tests, the Florisil and double reduction methods are suitable. The latter has the advantage of avoiding the use of pyridine.

SUMMARY

Slater and Morell's modification (9) of Najjar's fluorometric method (1) yields consistently good agreement in urinary riboflavin assays with microbiological assays with *Lactobacillus casei* and the Snell and Strong (4) medium, even for urines containing minute amounts of riboflavin. Assays by a Florisil adsorption procedure, including permanganate treatment of the eluates, are slightly higher. In terms of absolute amounts of riboflavin, these differences are small, but at a very low riboflavin level the percentage differences are high. Our modification (14) of the Hodson and Norris (2) double reduction method yields riboflavin values 0.1 to 0.2 mg. per day higher than do the microbiological assays, except at extremely low levels at which the smaller differences are probably associated with the purified nature of the diet.

High levels of urea in association with normal urinary riboflavin levels cause slight lowering of titrimetric microbiological assays, but do not affect turbidimetric measurements. Increases in urea-riboflavin ratios in low riboflavin urines increase this error in titrimetric assays. For this reason, as well as for speed and convenience, turbidimetric measurement is preferred.

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KINETICS AND INHIBITION OF CARBOXYPEPTIDASE ACTIVITY*

BY ELAINE ELKINS-KAUFMAN AND HANS NEURATH

(From the Department of Biochemistry, Duke University School of Medicine, Durham, North Carolina)

(Received for publication, May 22, 1948)

The specific activities of proteolytic enzymes toward synthetic peptide substrates have been commonly expressed as proteolytic coefficients, C , (2) which denote the first order reaction constant per mg. of enzyme N per cc. Independent determinations of the proteolytic coefficient for the hydrolysis of carbobenzoxyglycyl-L-phenylalanine (CGP) by carboxypeptidase, in 0.05 M substrate solutions (3-7), have yielded values ranging from $C = 10$ to $C = 13$. Reinvestigation of the mechanism of carboxypeptidase activity in this laboratory has revealed a large dependence of the apparent proteolytic coefficient of carboxypeptidase on the concentration of its specific substrate, CGP, the calculated coefficient increasing as substrate concentration decreases. Since at each substrate concentration the proteolytic coefficient was independent of enzyme concentration, the observed variations of C were ascribed to the substrate concentration dependence of the apparent first order reaction constant, k . An explanation of this phenomenon has required reconsideration of the kinetics of the hydrolysis by proteolytic enzymes and has led to the conclusion that simple first order reaction kinetics do not apply to the hydrolytic action of carboxypeptidase. The data presented in this paper sustain this conclusion and are in full accord with the theoretical considerations which are given in an introductory section of this paper.

In the course of these experiments the observation was made that certain D-amino acids, notably D-phenylalanine, are powerful inhibitors of carboxypeptidase. Inhibition is of the competitive type, and the kinetic constants of the system carboxypeptidase-CGP-phenylalanine have been determined from quantitative rate measurements.

General Considerations of Enzyme Kinetics

According to the classical theory of Michaelis and Menten (8) the first step in enzymatic reactions is the combination between free enzyme, E ,

* Part of a thesis to be submitted by Mrs. Elaine Elkins-Kaufman to the Graduate School of Duke University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Presented at the Thirty-ninth annual meeting of the American Society of Biological Chemists at Atlantic City, April 15-19, 1948 (1).

and substrate, S , to form the enzyme-substrate complex (ES). This, after activation, $(ES)^*$, decomposes into free enzyme and the reaction products, P .



Enzyme-substrate combination is governed by the dissociation constant of the complex, K_m , according to

$$K_m = \frac{(E)(S)}{(ES)} = \frac{(e - p)a}{p} \quad (2)$$

where e is the total concentration of enzyme, p that of the complex, and a the substrate concentration, at equilibrium (9). The rate of decomposition, v , of the enzyme-substrate complex is expressed by a velocity constant, k' ,

$$v = k' (ES) = k'p \quad (3)$$

where k' is proportional only to the concentration of the complex at any time, t . Since the concentration of the complex, p , is dependent on K_m and on substrate concentration, a , three cases may be considered to describe the over-all reaction kinetics. Of these, the first two are limiting conditions of the general relation which will be considered last.

Case I—If at equilibrium, p , the concentration of the complex, is negligibly small in comparison to the total enzyme concentration, e , and to the substrate concentration, a (equation (2)), K_m will be sufficiently high to force the equilibrium between free and combined enzyme far to the left of equation (1). Combination (9) of equations (2) and (3) yields

$$v = k'p = k' \frac{(e - p)a}{K_m} \quad (4)$$

If $e \gg p$, equation (4) reduces to

$$v = -\frac{da}{dt} = \frac{k'}{K_m} ea \quad (5)$$

which is the differential form of a first order reaction equation. Integration of equation (5) yields, accordingly,

$$k'et = 2.3 K_m \log \frac{a_0}{a} \quad (6)$$

According to Bergmann and coworkers (2), the hydrolysis of synthetic peptide derivatives by proteolytic enzymes follows first order reaction kinetics, which these authors have expressed in the form

$$kt = \log \frac{a_0}{a} \quad (7)$$

This first order reaction constant, k , is related to the proteolytic coefficient, C , by

$$C = \frac{k}{e} \quad (8)$$

where e is the total enzyme concentration in mg. of enzyme N per cc. Comparison of equations (7) and (8) with equation (6) shows the following interrelations¹ between k , C , k' , and K_m :

$$C = \frac{k}{e} = \frac{1}{2.3} \frac{k'}{K_m} \quad (9)$$

It is evident that a substrate concentration-independent first order reaction constant will be observed in all cases to which the assumptions for Case I apply, *i.e.* a high enzyme-substrate dissociation constant and low substrate concentration. Under these conditions, the rate of combination between enzyme and substrate will be the sole rate-determining step,¹ and the proteolytic coefficient a valid expression thereof.

Case II—If p and e are of similar magnitudes, and the substrate concentration is relatively high, the enzyme-substrate dissociation constant will be sufficiently low to cause all but a small fraction of the total enzyme to be in combination in the complex. Accordingly, for $e \simeq p$, equation (3) will reduce to

$$v = -\frac{da}{dt} = k'p = k'e = \text{constant} \quad (10)$$

and

$$k'et = (a_0 - a) \quad (11)$$

This is the expression of zero order kinetics, the rate-determining step being that of the conversion of the complex (ES) into the reaction products and free enzyme, or into some intermediary form (ES)*. This reaction mechanism has already been demonstrated for the specific esterase activities of certain proteolytic enzymes, *e.g.* trypsin (11) and carboxypeptidase

¹ The dissociation constant, K_m , is assumed to be equal to $(k_2 + k_3)/k_1$ (10), where k_1 is the specific velocity constant for the reaction $E + S \rightarrow (ES)$, k_2 that for the reverse reaction, and k_3 the specific velocity constant for the reaction $(ES) \rightarrow E + P$ (corresponding to k' of the denotations used in this paper). Accordingly, $k'/K_m = (k_3 \times k_1)/(k_2 + k_3)$. For the special case of $k_3 \ll k_2$, this reduces to k_1 which is identical with 2.3 times the first order reaction constant k used by Bergmann and coworkers (2).

(12). Theoretically, it should be possible to realize it for every enzymatic system provided the initial substrate concentration can be sufficiently raised to force the equilibrium between free and combined enzyme into the direction of the latter. Here, as in Case I, the reaction constant will be independent of substrate concentration provided no secondary effects, such as those arising from electrostatic interaction (13, 14), are operative.

Case III—The following general relations apply to all conditions in which the concentrations of free ($e - p$) and combined (p) enzyme at equilibrium are of comparable magnitudes. The concentration of the enzyme-substrate complex, p , will be determined by both the rate of formation and the rate of decomposition into free enzyme and reaction products.

Combination (9) of equations (2) and (3) yields

$$K_m = \frac{(e - p)ak'}{k'ae - av} \quad (12)$$

and

$$v = - \frac{da}{dt} = \frac{k'ae}{K_m + a} \quad (13)$$

Rearrangement and integration of equation (13) yields ^{2, 3}

$$k'et = 2.3K_m \log \frac{a_0}{a} + (a_0 - a) \quad (14)$$

Inspection of equation (14) reveals it to be a composite first order and zero order expression. Thus the first term on the right-hand side is identical with the right-hand term of equation (6), and the second term is identical with the right-hand term of equation (11). Since the first order term is independent of concentration, whereas the contribution of the zero order term increases in proportion with the increase in initial substrate concentration, a plot of the kinetic data according to conventional first order equations will yield non-linear relations. This is illustrated in Fig. 1 for a hypothetical case in which $K_m = 3 \times 10^{-2}$, $k' = 2$, and $e = 4 \times 10^{-4}$ mg.

² We are indebted to Dr. Irving Klotz for a discussion of these aspects of the problem.

³ The corresponding equation derived by Van Slyke (see Van Slyke, D. D., in Nord, F. F., and Werkman, C. H., *Advances in enzymology and related subjects*, New York, 2, 33 (1942)), with the symbols explained in foot-note 1, is

$$t = 1/k_1 \times \log a_0/a + (a_0 - a)/k_2.$$

That equation reduces to equation (14) of the present paper only if k_2 (defined in foot-note 1) is negligibly small in comparison to k' .

of N per cc. The initial substrate concentration has been varied within the range of 0.2 to 0.01 M. It will be noted that in regions of relatively high initial substrate concentration the velocity increases with time more rapidly than is required by first order kinetics, and that the initial slope of the curves increases as the initial substrate concentration decreases. In low initial substrate concentrations (0.02 and 0.01 M), the curves approximate a linear course and the slopes converge to a maximum value. These deviations from first order kinetics are to be expected from equation (14)

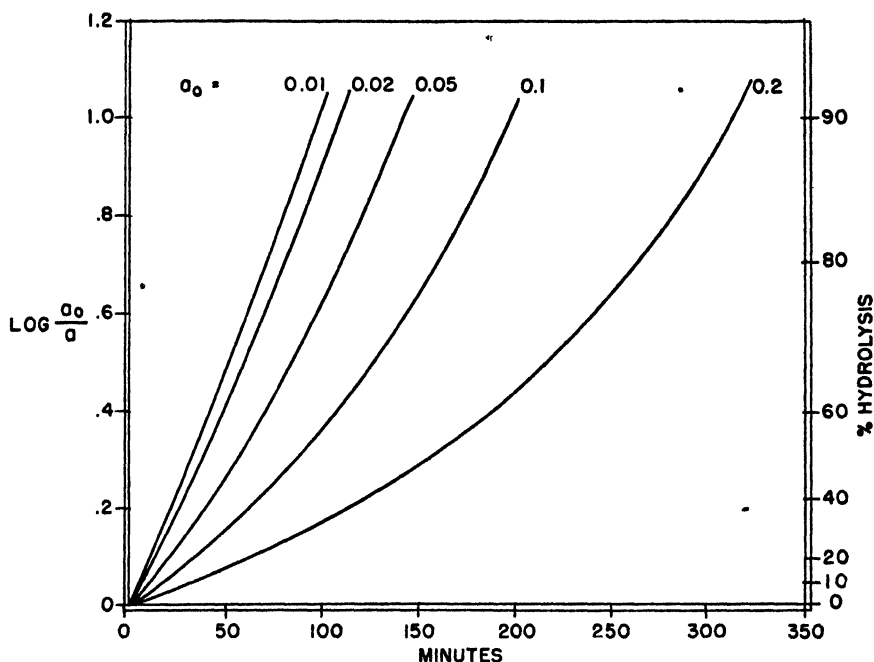


FIG. 1. Plot of the integrated Michaelis-Menten equation (equation (14)) according to first order reaction kinetics (equation (7)) in relation to the initial substrate concentration a_0 . K_m was assumed to be 3×10^{-3} , $k' = 2$, $e = 4 \times 10^{-4}$ mg. of enzyme N per cc., and $a_0 = 0.2$ to 0.01 M. For further explanations, see the text.

since the first order term will predominate as the initial substrate concentrations become relatively small. The habits of the curves are not merely due to the narrowing region on the time axis with decreasing substrate concentration. If the time required for 90 per cent hydrolysis is decreased to the same extent by increasing the enzyme concentration (the initial substrate concentration remaining constant), the initial slope will increase in proportion, but the curvature remains. This is illustrated in Fig. 2 in which the rate of the hydrolysis of the same hypothetical system is plotted

for a constant initial substrate concentration of 0.2 M, the enzyme concentration increasing over a 4-fold range.

It is evident, therefore, that in the general case interpretation of the kinetic data of enzymatic reactions by first order kinetics will lead to erroneous results unless the substrate concentration is decreased sufficiently to render the rate of combination of enzyme and substrate the rate-limiting step. In higher regions of substrate concentration, only the integrated form of the Michaelis-Menten equation (equation (14)) will apply.

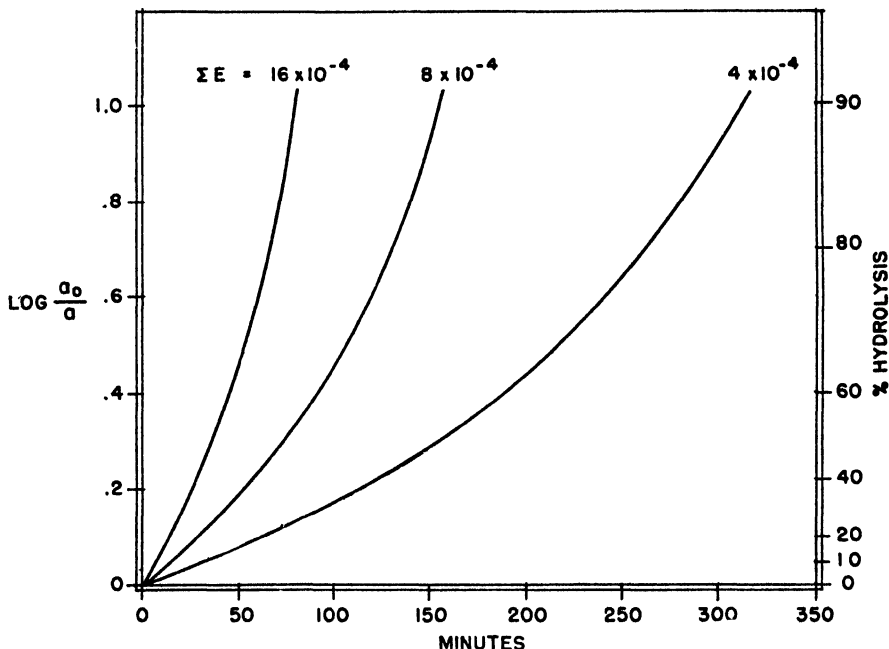


FIG. 2. Plot of the integrated Michaelis-Menten equation (equation (14)) according to first order reaction kinetics (equation (7)) in relation to enzyme concentration. K_m was assumed to be 3×10^{-2} , $k' = 2$, the initial substrate concentration $a_0 = 0.2$ M. Enzyme concentration (denoted in this graph as ΣE) is 4 to 16×10^{-4} mg. of N per cc.

Determination of K_m and k' —Lineweaver and Burk (15) have given equations for the calculation of K_m from the initial reaction velocity. Of these, the following equation has been used in this work.⁴

$$\frac{a}{v} = \frac{K_m}{V_{\max.}} + \frac{1}{V_{\max.}} \quad (15)$$

Eadie (16) has proposed the equation $v = V_{\max.} - K_m \times v/a$ which has the advantage that the slope, K_m , is the regression coefficient and that the two constants appear in separate terms. This equation has likewise been used in this work for the determination of K_m .

where v was taken as the moles per liter of substrate hydrolyzed during the first 30 minutes of hydrolysis (v_{30}), when the enzyme concentration was sufficiently low for not more than about 30 per cent of the total substrate to be hydrolyzed during that period. These values were then corrected to a common enzyme concentration of 4×10^{-4} mg. of N per cc. V_{max} is the maximum velocity and is equal to $k' \times e$ (9).

The integral reaction constant k' , may be determined by any one of the following three methods.

1. At any initial substrate concentration, $e \times t$ is plotted against the expression on the right-hand side of equation (14), K_m having been previously determined as described above. The slope of the resulting straight line is equal to k' .

2. Since in equation (15), V_{max} is equal to $k'e$ (9), k' can be determined directly from a plot according to equation (15), where the slope is equal to $1/V_{max}$ when a/v is plotted along the axis of the ordinates and a along the axis of the abscissas. If $v = v_{30}$, V_{max} calculated in this fashion has to be corrected to the maximum velocity per minute of hydrolysis.

3. In regions of low initial substrate concentrations, in which the calculated first order reaction constant, k , becomes independent of substrate concentration, k' may be calculated directly from k , K_m , and e , according to equation (9).

EXPERIMENTAL

Enzyme—Six times recrystallized carboxypeptidase was prepared as previously described (6, 7).

Substrates and Inhibitors—The DL and L forms of carbobenzoxyglycyl-phenylalanine (CGP) were prepared as already described (7). The D isomers of tyrosine, histidine, isoleucine, alanine, and lysine were from the collection of Dr. F. Bernheim, who kindly placed these at our disposal. D-Phenylalanine was received through the courtesy of Dr. W. H. Stein.

Methods—Enzymatic measurements were carried out at 25° in a 0.04 M phosphate buffer, pH 7.5, containing 0.1 M LiCl. Enzyme solutions were prepared daily from stock solutions containing about 0.2 to 0.5 mg. of N per cc. The latter was prepared about every 3rd day from a stock suspension of crystals. In the earlier part of this work, the progress of hydrolysis was determined with the manometric ninhydrin method (17) on aliquots varying from 0.2 to 0.8 cc., depending on initial substrate concentration. In the later phase, the colorimetric ninhydrin method, as described by Schwert (18), was used. Control measurements have shown that these two methods give strictly comparable results. Initial substrate concentrations were determined by nitrogen analyses.

Results

Dependence on Substrate Concentration

The first order reaction constant, k , for the hydrolysis of CGP by carboxypeptidase at constant enzyme concentration increases with decreasing substrate concentration. This effect has been observed with both the racemic form of the substrate and with the L isomer. The results are illustrated in Fig. 3 in which the proteolytic coefficient, C , is plotted along

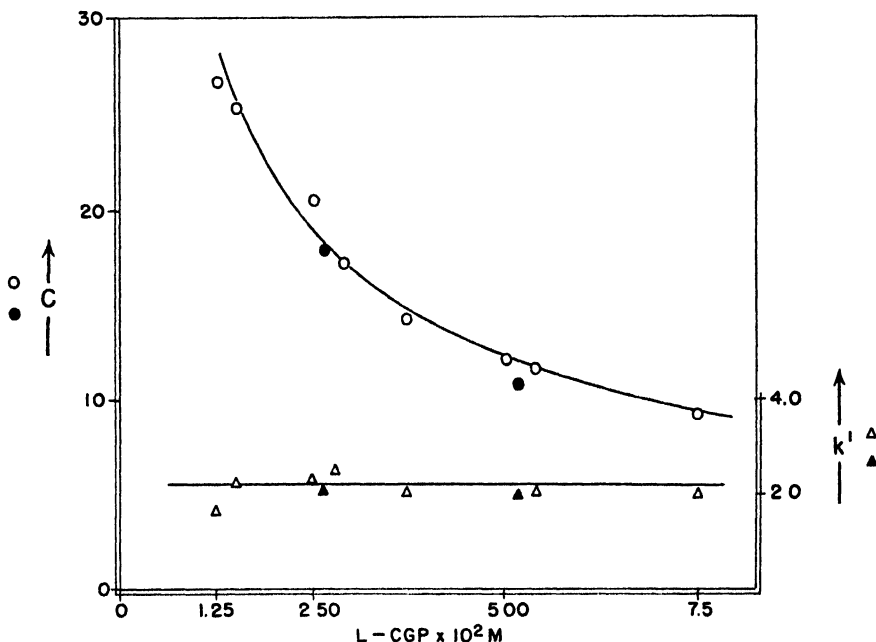


FIG. 3. Plot of the apparent proteolytic coefficient, C , and of the integral reaction constant, k' , against initial concentration of the substrate, L-CGP. $\circ$ and Δ = DL-CGP; $\bullet$ and $\blacktriangle$ = L-CGP. C values were calculated from equations (7) and (8), and k' values from equation (14), with the K_m values given in the text.

the left-hand ordinate and the initial substrate concentration (with respect to the L isomer) along the abscissa. Within the range of substrate concentration investigated, the proteolytic coefficient varies from a lower limit of $C = 9$ at 0.075 M substrate to an upper limit of C of about 28 at 0.0125 M substrate. As shown in Fig. 3, the results obtained with L-CGP follow the relation plotted for the racemic form. Since, in agreement with previous work (3, 7), the proteolytic coefficient was found to be independent of enzyme concentration when substrate concentration was held constant, supporting data for this finding are not reproduced herein.

Since, according to theory, a first order reaction constant should be independent of initial substrate concentration, the kinetics of hydrolysis of CGP by carboxypeptidase were reinvestigated. In these experiments, hydrolysis was followed up to above 80 per cent of completion, since in the earlier phase of reaction the order of reaction cannot always be unequivocally determined. The results of two of several detailed measurements are

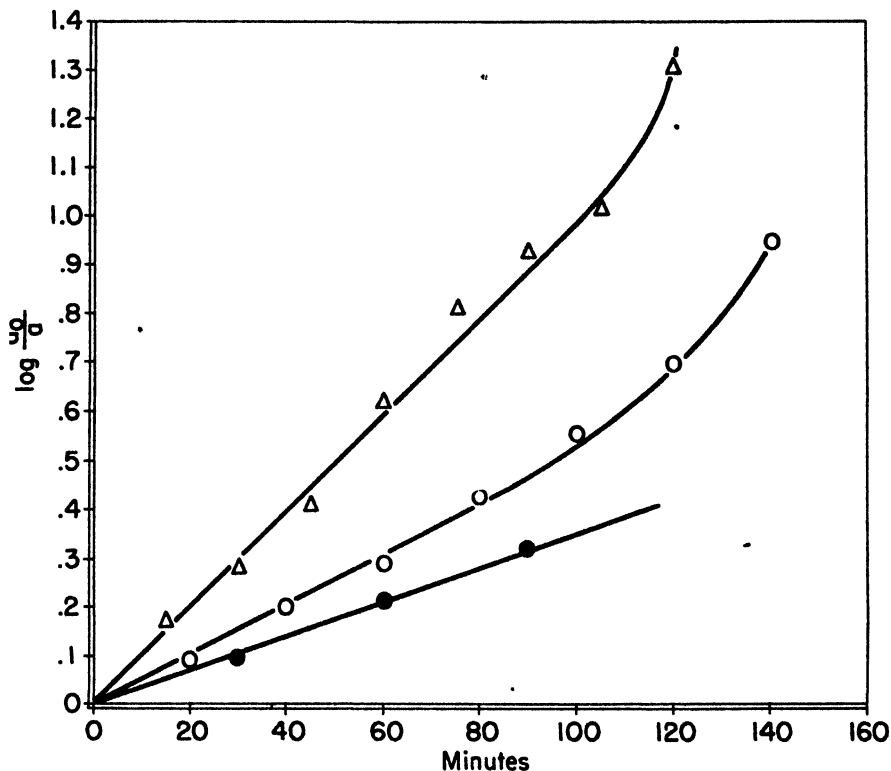


FIG. 4. First order reaction plot of the hydrolysis of DL-CGP by carboxypeptidase, corrected to an enzyme concentration of 4×10^{-4} mg. of N per cc. The substrate concentrations (with respect to L-CGP) were, respectively, 0.0128 M (Δ), 0.0548 M ($\circ$), and 0.0753 M ($\bullet$).

given in Fig. 4 for 0.0548 M and 0.0128 M DL-CGP (concentration with respect to the L isomer), corrected to a common enzyme concentration of 4×10^{-4} mg. of N per cc. It will be noted that the curves deviate from the linear course required by first order reaction kinetics. It is also apparent from the results given in Fig. 4 that the first order reaction constant, as expressed by the slope of the seemingly linear portion of these curves,

increases as substrate concentration decreases. For purposes of comparison, the initial rate of hydrolysis for a higher initial substrate concentration (0.0753 M) is also plotted in Fig. 4.

The results of kinetic measurements in substrate concentrations lower than those given in Figs. 3 and 4 did not yield to unequivocal interpretation. In this low substrate concentration range, the deviations from first order kinetics were in the opposite direction from those observed in the higher concentration range, and the course of reaction appeared to simulate second order kinetics. These deviations were observed below 0.0125 M (with respect to the L isomer) for DL-CGP, and below 0.025 M for L-CGP.

Determination of K_m —In accordance with the theoretical considerations discussed in a preceding paragraph, a complete resolution of the reaction kinetics of the present system requires determination of the enzyme-substrate dissociation constant, K_m . This has been done according to equation (15)⁴ by plotting a/v_{30} against a , where a is the initial substrate concentration. From the straight line, calculated by the methods of least squares, the slope and intercept were determined, yielding values for K_m and $V_{\max}^{30}$, the enzyme-substrate dissociation constant and the maximum velocity, respectively. The values obtained for DL-CGP are, respectively, $K_m = 3.3 \times 10^{-2}$ and $V_{\max}^{30}$ ($e = 4 \times 10^{-4}$ mg. of N per cc.) = 2.4×10^{-2} . The corresponding values for L-CGP are approximately the same, *i.e.* 3.7×10^{-2} and 2.2×10^{-2} , respectively.

Determination of k' —The integrated form of the Michaelis-Menten equation (equation (14)) yields a constant, k' , which is independent of time of hydrolysis, of substrate concentration, and of enzyme concentration. It has been shown in Figs. 3 and 4 that in accordance with theoretical considerations (see above) the first order reaction constant, k , at constant enzyme concentration is dependent on substrate concentration and that in relatively high substrate concentrations this "constant" increases with increasing time of hydrolysis. The detailed results given in Table I demonstrate more clearly the deviations of the first order reaction constants from theoretical requirements, for both DL- and L-CGP.

For each time increment, the first order reaction constant was calculated according to equation (7) and expressed as an apparent proteolytic coefficient by dividing by the enzyme concentration. It will be noted that in the higher substrate concentrations (about 0.05 M with respect to the L isomer) the apparent proteolytic coefficient increases with increasing time of hydrolysis, whereas more nearly constant, though higher, values were obtained in each of the experiments with lower initial substrate concentration. In contrast, no significant trend resulted when the data were interpreted according to the integrated equation (equation (14)), the calculated constants being essentially independent of time, and independent also of

initial substrate concentration. Comparison of the apparent proteolytic coefficients with the integrated reaction constant, k' , over the entire substrate concentration range investigated is made graphically in Fig. 3, which shows k' to remain essentially constant.

Inhibition by D-Phenylalanine

In order to elucidate further the mechanism of the hydrolysis of CGP by carboxypeptidase, the influence of the reaction products on the rate was investigated. Neither carbobenzoxyglycine nor L-phenylalanine had any inhibitory effect whatsoever. However, D-phenylalanine was found

TABLE I
Representative Kinetic Data for Hydrolysis of CGP by Carboxypeptidase

System	<i>t</i>	Hydrolysis	$\frac{k}{e}$	k'	System	<i>t</i>	Hydrolysis	$\frac{k}{e}$	k'
	min.	per cent				min.	per cent		
DL-CGP 0.055 M (L-), $e = 4.13 \times 10^{-4}$	20	21.6	12.1	2.3	DL-CGP 0.0128 M (L-), $e = 2.38 \times 10^{-4}$	30	30.6	22.2	2.2
	40	37.8	12.5	2.2		45	41.1	21.5	2.1
	60	49.8	12.2	2.0		60	56.8	25.5	2.4
	80	63.8	13.6	2.1		75	66.6	26.8	2.5
	100	73.0	13.7	2.0		90	71.4	25.4	2.4
	120	81.2	14.6	2.0		105	76.0	25.1	2.3
	140	89.5	16.8	1.8		120	83.4	27.3	2.5
L-CGP 0.0517 M, $e = 4.33 \times 10^{-4}$	20	16.2	9.0	1.7	L-CGP 0.0258 M, $e = 3.38 \times 10^{-4}$	20	24.8	18.3	2.3
	40	33.4	10.3	1.8		35	39.6	18.4	2.2
	60	52.0	12.4	2.0		50	49.5	17.4	2.1
	80	67.7	14.2	2.1		65	58.0	17.2	2.0
	100	77.5	14.9	2.1		80	67.0	17.9	2.0
	120	86.0	16.3	2.1		95	78.3	20.6	2.2
	140	89.0	15.8	2.1					

k = first order reaction constant according to equation (7). e = mg. of enzyme N per cc. k' = reaction constant according to equation (14).

to be a potent inhibitor. It was first thought that the dependence of reaction rate on substrate concentration and the inhibition by D-phenylalanine were interrelated phenomena as a result of optical inversion of L-phenylalanine during enzymatic hydrolysis. This hypothesis was discredited by experiments in which D-amino acid oxidase was added to the enzymatic digest of L-CGP, which failed to reveal any measurable oxygen uptake.⁵ Since control experiments indicated that carbobenzoxyglycine caused partial inhibition of D-amino acid oxidase activity, oxidation was

⁵ We are indebted to Dr. F. Bernheim for performing the measurements involving D-amino acid oxidase. Fresh rat kidney slices were used as a source of this enzyme.

also measured with the isolated end product, phenylalanine, as substrate. The results were entirely negative.

A series of extensive kinetic measurements was made to elucidate the mechanism of inhibition by D-phenylalanine. By means of accepted pro-

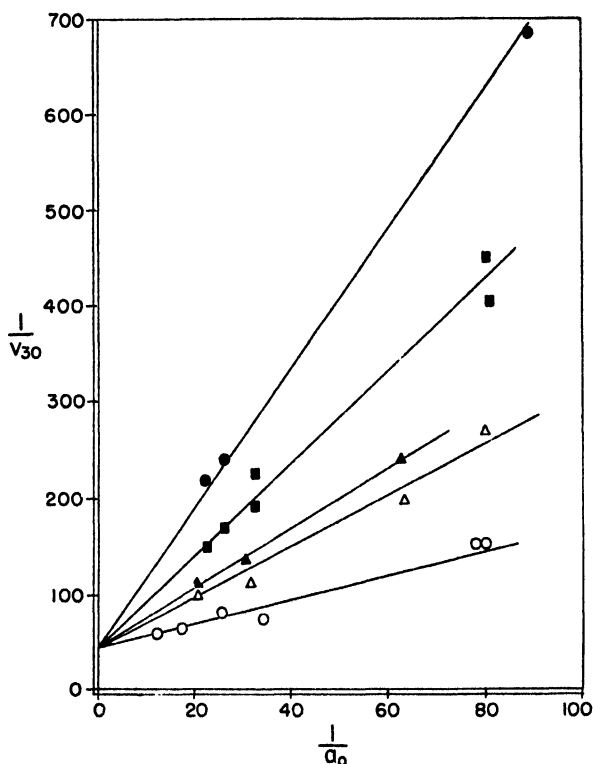


FIG. 5. Competitive inhibition of carboxypeptidase activity by D-phenylalanine and D-histidine. $1/v_{30}$ is plotted along the axis of the ordinate, and $1/a_0$ along the axis of the abscissa, according to equation (16). v_{30} is the initial reaction velocity expressed in moles per liter of L-CGP hydrolyzed during the first 30 minutes, corrected to an enzyme concentration of 4×10^{-4} mg. of N per cc. a_0 is the initial concentration of the substrate, DL-CGP (with respect to the L isomer). $\circ$ = no added inhibitor; $\triangle$ = in the presence of 0.01 M D-histidine; the concentrations of added D-phenylalanine are denoted by $\blacktriangle$ = 0.0025 M, $\blacksquare$ = 0.005 M, and $\bullet$ = 0.01 M. The straight lines were calculated by the method of least squares.

cedures (15) the influence of both variables, *i.e.* substrate concentration and inhibitor concentration, on the initial reaction velocity was separately determined. The results are given in Fig. 5 in which $1/v_{30}$ is plotted against $1/a_0$, where, as before, v_{30} is the initial reaction velocity after 30 minutes of hydrolysis, corrected to a constant enzyme concentration of 4×10^{-4} mg.

of N per cc., and a , the initial substrate concentration. The three straight lines, calculated by the method of least squares and corresponding, respectively, to 0.01, 0.005, and 0.0025 M D-phenylalanine, intersect at a common intercept, identical with that obtained in the absence of D-phenylalanine. This is characteristic of competitive inhibition (15). From the slope of these curves in comparison to that of the control experiment (no added inhibitor), the enzyme-inhibitor dissociation constant, K_I , was calculated, according to the relation (15)

$$\frac{1}{v} = \frac{1}{V_{\max.}} \left[K_m + \frac{K_m(I)}{K_I} \right] \frac{1}{a} + \frac{1}{V_{\max.}} \quad (16)$$

where (I) is the inhibitor concentration.

The calculated value of K_I is 2×10^{-3} , which is about 17 times lower than that of the enzyme-substrate dissociation constant, K_m , which is 3.3×10^{-2} . In the presence of a competitive inhibitor, the integrated form of the Michaelis-Menten equation assumes the form

$$k'el = 2.3 \left[K_m + \frac{K_m(I)}{K_I} \right] \log \frac{a_0}{a} + (a_0 - a) \quad (17)$$

The integrated constant, k' , accordingly, should be identical with that calculated from measurements in the absence of the inhibitor, and should likewise be independent of time and substrate concentration. Representative plots of time against F' , where F' is the expression on the right of equation (17) divided by e , the enzyme concentration in mg. of N per cc., are shown in Fig. 6. It will be noted that the values obtained from experiments with varying substrate and inhibitor concentrations follow closely a common straight line whose slope is $k' = 2.1$, which is identical with the mean value obtained in the absence of inhibitor.

Inhibition by D-Histidine and Other D-Amino Acids—Although phenylalanyl peptides are the most specific substrates of carboxypeptidase, certain N-acyl derivatives of other amino acids, such as tyrosine (3), leucine, and isoleucine (19), are split as well, though to a considerably lesser degree. It seemed of interest, therefore, to test the inhibitory activity of D isomers of other amino acids in comparison to that of D-phenylalanine.

D-Histidine was found to be considerably inhibitory. The results obtained with solutions containing varying concentrations of DL-CGP and 0.01 M D-histidine are plotted in Fig. 5. Inhibition may be seen to be of the competitive type, the enzyme-inhibitor dissociation constant, K_I , being of the same order of magnitude as the enzyme-substrate dissociation constant, K_m , i.e. 2×10^{-2} as compared to 3.3×10^{-2} . The integral reaction constant, k' , calculated according to equation (17), is approximately the same as that obtained in the absence of an inhibitor, i.e. 1.9 as compared to 2.1.

Exploratory measurements on the inhibitory activity of D-alanine, D-isoleucine, and D-lysine were limited to rate measurements at a single concentration of DL-CGP, *i.e.* 0.0125 M (with respect to the L isomer), the concentration of the D-amino acids being 0.01 M. The data are given in Table II, and are expressed as apparent proteolytic coefficients, C . It will be noted that the inhibitory activities of D-alanine and D-isoleucine are

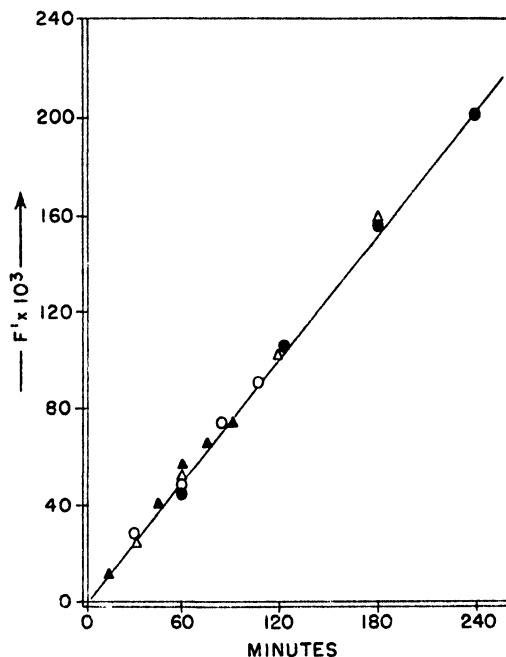


FIG. 6. A plot to demonstrate the validity of equation (17). F' is the right-hand expression of equation (17), divided by the enzyme concentration e . The symbols denote the following: ● = 0.045 M DL-CGP (with respect to the L isomer) + 0.01 M D-phenylalanine; ○ = 0.0112 M DL-CGP (with respect to the L isomer) + 0.01 M D-phenylalanine; △ = 0.0305 M DL-CGP (with respect to the L isomer) + 0.005 M D-phenylalanine; ▲ = 0.048 M DL-CGP (with respect to the L isomer) + 0.0025 M D-phenylalanine. For values of K_m and K_I , see Table III.

equal and somewhat less than that of D-histidine. D-Lysine has no measurable inhibitory activity whatsoever.⁶

A summary of the characteristic kinetic constants of carboxypeptidase is given in Table III.

⁶ Preliminary experiments revealed that D-tyrosine is likewise inhibitory. Because of the limited solubility of this amino acid, experiments over a range of D-tyrosine concentrations could not be performed.

DISCUSSION

The kinetics of the hydrolysis of CGP by carboxypeptidase follows, within the limits of the experimental error, the course described as Case III in the preceding theoretical considerations. Because of the relatively low enzyme-substrate dissociation constant, K_m , interpretation of the results by first order reaction equations yields a substrate concentration-dependent first order reaction "constant" which increases with decreasing initial substrate concentration. Moreover, at any given initial substrate concentration the rate of hydrolysis increases more rapidly with time than is

TABLE II
Effects of Added D-Amino Acids (0.01 M) on Hydrolysis of 0.0125 M L-CGP by Carboxypeptidase

D-Amino acid	C*	D-Amino acid	C*
None	27	Isoleucine	17
Lysine	25	Histidine	13
Alanine	17	Phenylalanine	4.5

*C denotes the apparent proteolytic coefficient calculated on the assumption of first order reaction kinetics.

TABLE III
Summary of Kinetic Constants of Carboxypeptidase-CGP System

Substrate	Inhibitor	$K_m \times 10^3$	$K_I \times 10^3$	k'	C*
DL-CGP	None	3.3		2.1	27.8
L-CGP	"	3.7		2.2	28
DL-CGP	D-Phenylalanine		0.33	2.1	
"	D-Histidine		2.0	1.9	

* Maximum proteolytic coefficient calculated from k' and K_m according to equation (9).

required by first order reaction kinetics. This deviation is more apparent the higher the substrate concentration. These anomalies follow directly from the characteristics of the integrated Michaelis-Menten equation (equation (14)) which reduces to a first order reaction equation only when (a) K_m is sufficiently high to render the equilibrium concentration of the enzyme-substrate complex (p) small in comparison to the total enzyme concentration (e), or (b) the substrate concentration becomes sufficiently small to render the first term of equation (14) predominant in comparison to the zero order term.

The integrated reaction constant k' is independent of both substrate and

enzyme concentration and characteristic only of the enzymatic system. This is as it should be for a truly catalytic reaction.

The proteolytic coefficient of the system carboxypeptidase-CGP previously reported to be within the range $C = 10$ to 13 (3-7) is an equivocal value. Its true limiting value, determined either from rate measurements in low substrate concentrations (0.01 to 0.02 M with respect to L-CGP) or from equation (9), is about $C = 28$.

Since even the apparent first order reaction constant is dependent on substrate concentration, the concept of homospecificity of proteolytic enzymes, introduced by Irving, Fruton, and Bergmann (2), requires reconsideration. According to this concept, several enzymes may resemble one another with regard to side group and backbone specificity such that the ratio of the proteolytic coefficient of the enzymes toward two or more substrates will be the same for all homospecific enzymes. For instance, carboxypeptidases isolated from beef spleen, beef kidney, swine kidney, and beef pancreas have been reported to be homospecific when tested against L-CGP and carbobenzoxyglycyl-L-tyrosine, the ratio of the corresponding proteolytic coefficients being 1.6 to 1.8 for all four enzymes (20). Similarly, various trypsinases were reported to be homospecific when tested against benzoylargininamide and benzoyllysineamide as substrates (20). Since, however, the proteolytic coefficient is of limited validity (see above) to express enzyme activities and, moreover, depends on both the affinity of the substrate for the enzyme (K_m), and on the rate of decomposition of the complex (k'), rigorous proof for the reality of the concept of homospecificity requires determination of the constants K_m and k' for each enzyme-substrate system.

It has been recently shown (7) that, contrary to earlier claims (19), the concept of antipodal inhibition does not apply to the system carboxypeptidase-CGP. Thus, while the D isomer of this specific substrate is not hydrolyzed, it does not inhibit the action of carboxypeptidase toward the corresponding L antipode. This observation is further sustained by the results of the present investigation which show that over a wide range DL- and L-CGP obey the same substrate concentration dependence of apparent proteolytic coefficients (Fig. 3) and that they likewise have the same kinetic characteristics, K_m and k' . However, other apparent differences were noted. Deviations from first order kinetics in the direction of apparent second order kinetics were observed in regions of low substrate concentrations, *i.e.* below 0.0125 M for DL-CGP (with respect to the L isomer) and below 0.025 M for L-CGP. A resolution of these apparent discrepancies has to await further investigation.

Although D-CGP has no inhibitory effect on the enzymatic hydrolysis of the L isomer, the D isomer of the free amino acid, phenylalanine, is

strongly inhibitory. Since the inhibition is of the competitive type, it has to be concluded that the L peptide and the D-amino acid are bound by the same active centers on the enzyme surface. Comparison of the corresponding dissociation constants, K_m and K_i , shows that the affinity of the enzyme for the amino acid is about 17 times greater than for the peptide. Whereas it has been previously postulated (3, 19) that the free carboxyl group as well as the sensitive peptide group is a requisite structural element of specific substrates for carboxypeptidase, the present evidence indicates that the latter is not only dispensable for combination between the enzyme and the D-amino acid, but actually prevents it. Although other D-amino acids are likewise inhibitory, their activity appears to be related to the substrate specificity of the corresponding L peptides (19).⁷ This may be simply related to the stereochemical characteristics of the amino acid residues relative to the steric requirements of the active centers on the enzyme surface. It may also suggest, however, that a second point of combination between enzyme and substrate (or inhibitor) is located somewhere on the non-polar amino acid residue and that combination also occurs by non-polar interaction.

The present discovery of a specific inhibitor for carboxypeptidase of relatively simple structural characteristics, *i.e.* D-phenylalanine, appears to pave the way for the elucidation of the nature of the groups and the stereochemical arrangements required for specific combination between enzyme and inhibitor, or substrate. This is now being done in this laboratory by a systematic study in which the essential contribution to combination of each of the four radicals attached to the α -carbon atom is tested by replacement by structurally analogous or dissimilar groupings.

No attempt will be made to reconcile the present findings with those of Stahmann, Fruton, and Bergmann (19) according to which not only D-CGP but also other, relatively enzyme-resistant, peptides of both the L and D series, as well as carbobenzoxyglycylglycine, inhibit the enzymatic activity of carboxypeptidase toward L-CGP. It has already been stated, and again shown in this paper, that we were unable to duplicate their findings on the inhibitory activity of D-CGP. Carbobenzoxyglycine is entirely devoid of inhibitory activity, as is carbobenzoxyglycylglycine.⁸ Further investigation is required in order to corroborate the reported inhibitory activity of the other peptides of Stahmann and coworkers (19).

⁷ Experiments on the splitting of N-substituted histidine derivatives by carboxypeptidase are in progress and will be reported elsewhere.

⁸ The presence of 0.033 M and 0.05 M carbobenzoxyglycylglycine in reaction mixtures containing, respectively, 0.03 M and 0.04 M DL-CGP (concentrations with respect to the L isomer) failed to exert any measurable influence on the rate of hydrolysis of CGP by carboxypeptidase.

The physiological significance of the inhibitory activity of certain D-amino acids toward carboxypeptidase remains to be evaluated. Although the occurrence of D-amino acid oxidase is beyond dispute, the origin and rôle of D-amino acids in intermediary protein metabolism remain obscure (21). The discovery of the inhibition of carboxypeptidase by D-amino acids may play a part in this ill understood system.

This work has been supported by the Rockefeller Foundation, by the United States Public Health Service, National Institute of Health, Division of Grants and Fellowships, and by the Duke University Research Council.

SUMMARY

In confirmation of theoretical considerations, given in an introductory section of this paper, it has been shown that the hydrolysis of carbo-benzoylglycylphenylalanine (CGP) by carboxypeptidase does not follow simple first order reaction kinetics. The increase of approximated first order reaction constants with decreasing substrate concentration is related to the change in the equilibrium between the free enzyme and the enzyme-substrate complex with varying substrate concentration. Integration of the Michaelis-Menten equation yields a reaction constant, k' , which is independent of both enzyme and substrate concentrations and characteristic of the reaction system. This constant is related to the proteolytic coefficient by the enzyme-substrate dissociation constant ($K_m = 3.3 \times 10^{-2}$) and yields a maximum proteolytic coefficient of $C = 28$ in low substrate concentrations.

It has been shown that both K_m and k' are identical for solutions containing L-CGP alone or in the presence of D-CGP, thus confirming the previous observation that the concept of antipodal inhibition does not apply to this system.

The activity of carboxypeptidase toward CGP is competitively inhibited by certain D-amino acids. Of these, D-phenylalanine is the most potent inhibitor, the corresponding enzyme-inhibitor dissociation constant, K_i , being about 17 times lower than the enzyme-substrate dissociation constant.

The significance of the present findings in relation to the concept of the homospecificity of proteolytic enzymes and to the structural requirements of specific substrates of carboxypeptidase has been discussed.

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EVIDENCE AGAINST THE OCCURRENCE OF A TRICARBOXYLIC ACID CYCLE IN *AZOTOBACTER AGILIS**

By J. L. KARLSSON AND H. A. BARKER

(From the Division of Plant Nutrition, University of California, Berkeley)

(Received for publication, May 21, 1948)

The tricarboxylic acid cycle theory of the mechanism of pyruvate and acetate oxidation has been well substantiated by work on animal tissues in recent years (11). However, little evidence is available to support the existence of such a cycle in bacteria, and certain reports are in contradiction to the theory. For instance, Lenti (7) was able to demonstrate inhibition of succinate oxidation in *Escherichia coli* by malonate, while the oxidation of pyruvate was not affected.

We have investigated the occurrence of the tricarboxylic acid cycle in *Azotobacter agilis* by two independent methods and have obtained evidence that it is not operative in this organism.

The first method is based upon the fact that the oxidative enzymes of *Azotobacter* are generally adaptive; *i.e.*, they are formed only in response to the homologous substrate (2, 3). As shown by the senior author (5) and, independently, by Stanier (8), the occurrence of adaptive enzymes can be employed to investigate the existence of suspected metabolic pathways, since the organism will as a rule be adapted to attack a given compound only if this substance has been added to the culture or formed as an intermediate during the oxidation of the growth substrate. If an enzyme attacking a certain compound is not present in cells grown on a different substrate, it can be concluded that the compound has not been formed in an appreciable quantity during the metabolism of the growth substrate. Conversely, the presence of the enzyme indicates that the compound may have been formed as an intermediate during growth.

The second method involves the use of a radioactive isotope. One procedure is based upon an isotope dilution principle. A substrate is oxidized in the presence of a postulated intermediate labeled with C^{14} . After the oxidation the intermediate is reisolated, and its initial and final activities are compared. A reduction in specific activity provides proof that the postulated intermediate has been formed in the oxidation. If no dilution is observed, the opposite conclusion may be accepted, provided there is evidence that the labeled compound can penetrate the cell wall. A similar approach involves the oxidation of a radioactive substrate in the

* Supported in part by a grant from the United States Public Health Service.

presence of a postulated intermediate to see whether any of the activity becomes incorporated in the latter.

Materials and Methods

The organism employed in this work was *Azotobacter agilis*, strain 4.4, obtained from Professor C. B. van Niel. One experiment was carried out with an artificially induced variant of this organism, designated A13 (5, 6).

The growth medium had the following composition, made up in distilled water: 0.5 to 1.0 per cent substrate, 0.1 per cent K_2HPO_4 , 0.02 per cent $MgSO_4$, 0.01 per cent $CaSO_4$, 0.003 per cent $FeSO_4$, 0.0002 per cent $NaMoO_4$; pH adjusted to 7.2 with HCl. The incubation temperature was 28–30°. To insure uniform and rapid growth the cultures were agitated on a horizontal shaker.

Respiration was measured by the Warburg technique. The cells for manometric experiments were harvested after 3 days incubation, washed, and resuspended in M/30 phosphate buffer, pH 7.2, containing 0.005 M $CaSO_4$. The rate of oxygen uptake was determined at 26°, with KOH to absorb carbon dioxide. The rates are expressed as Q_{O_2} (N) values. Total nitrogen was estimated by the micro-Kjeldahl method.

Succinic acid labeled with C^{14} in the carboxyl groups was synthesized by the biological method of van Niel *et al.* (10), with the ciliate *Tetrahymena gelii*. The acid was isolated from cell suspensions by ether extraction, and contaminating substances were removed by permanganate oxidation, steam distillation, and basic ether extraction. The resulting material was finally sublimed at 150° under 2 mm. of mercury pressure. Total succinate was estimated by the succinoxidase method. Doubly labeled acetic acid was prepared by the use of *Clostridium acetivum*.¹ Acetic acid was isolated by steam distillation and estimated by titration. The β -carboxyl group of oxalacetic acid was obtained by the aniline citrate method (9). Radioactivity measurements were made with a Geiger-Müller counter as described by Kamen (4).

Results

Adaptation Experiments—Our experiments have shown that the oxidative enzymes of *Azotobacter agilis* involved in the decomposition of the following compounds are adaptive: glucose, gluconate, *cis*- and *trans*-aconitate, α -ketoglutarate, succinate, fumarate, malate, pyruvate, tartrate, malonate, and ethyl alcohol. Cells grown on acetate show lag periods for all these compounds, but regardless of the growth substrate,

¹ Volcani, B., and Barker, H. A., unpublished data.

acetate itself never requires adaptation, probably because it is formed during the oxidation of all other substrates, as indicated by independent evidence (5).

Table I shows the initial respiratory rates for a series of compounds involved in the tricarboxylic acid cycle by cells grown on the same compounds. Read horizontally, Table I shows that each growth substrate causes complete adaptation to all the more oxidized members, but does not in general cause adaptation to more reduced compounds. There are, however, exceptions to this generalization in the case of succinate. Read vertically, Table I demonstrates that the rate of respiration is slower when the substrates below the homologous one are used for growth, indicating that the oxidation of the lower compounds does not result in formation of the higher compounds, as the tricarboxylic acid cycle theory would require.

TABLE I
Respiration Rates in Relation to Growth Substrate^a

Growth substrate	Q_{O_2} (N) $\times 10^{-2}$ for first 20 min.						
	α -Keto-glutarate	Succinate	Fumarate	Malate	Pyruvate	Acetate	Endo-genous
α -Ketoglutarate.....	10	25	17	25	28	32	0
Succinate.....	2	30	26	26	25	30	2
Fumarate.....	0	31	31	34	29	33	0
Malate.....	0	17	9	23	29	47	0
Pyruvate.....	0	16	9	19	26	28	0
Acetate.....	2	3	2	9	7	29	2

The adaptation of cell suspensions of *Azotobacter agilis* to a new substrate occurs rather rapidly. In Table I the Q_{O_2} (N) values obtained during the first 20 minutes are used because after a somewhat longer exposure to a substrate (30 to 45 minutes) a significant adaptation occurs. Respiration curves for succinate- and acetate-grown cells on several substrates are shown in Figs. 1 and 2 as examples of how adaptation to all compounds eventually takes place. It is also clear from these graphs how acetate-grown cells require adaptation to all the other compounds, whereas succinate-grown cells require adaptation to α -ketoglutarate, but not to fumarate, malate, pyruvate, or acetate.

Isotope Experiments—With the aid of succinate labeled in the carboxyl groups with C^{14} experiments were made to determine directly whether succinate is formed from acetate. In Experiment I cells were used which were not adapted to succinate, whereas in Experiment II cells adapted to both succinate and acetate were used so as to insure entrance of the

succinate into the cells. The initial and final activities of the succinate were determined, and only an insignificant dilution was found, as shown

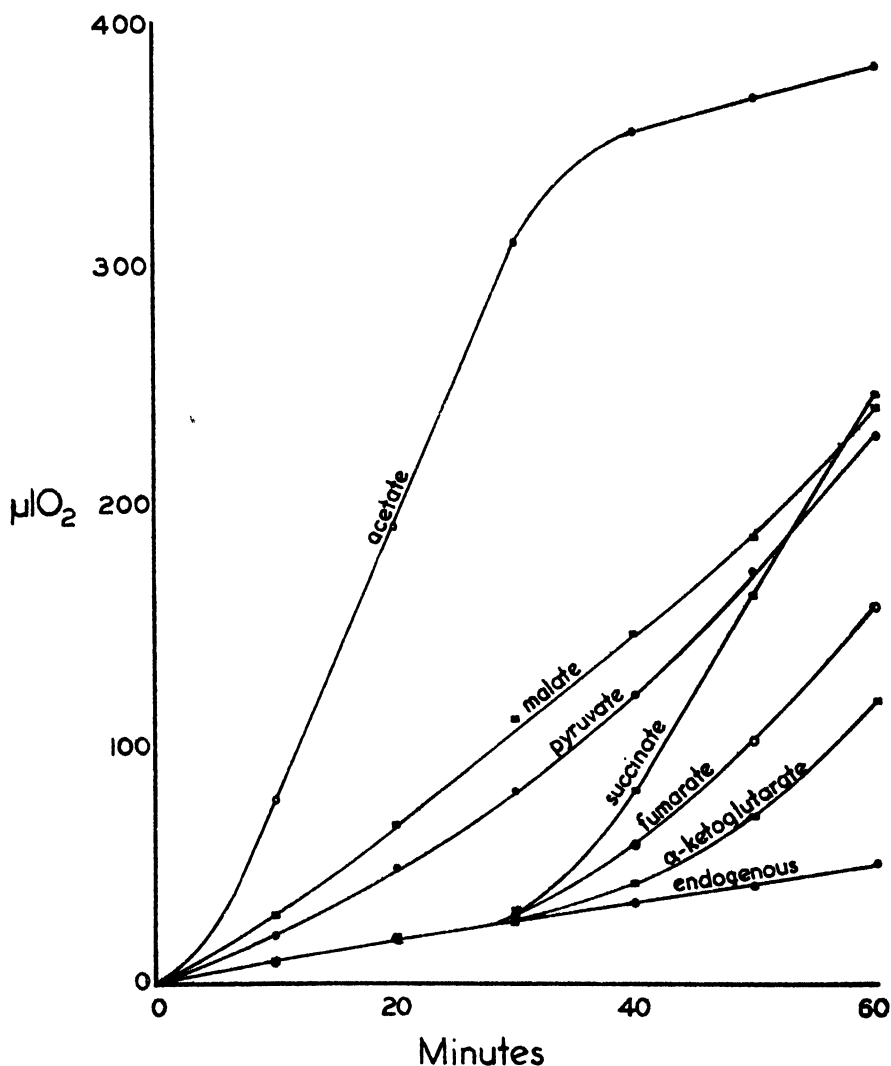


FIG. 1. Respiration curves for acetate-grown cells. Theoretical oxygen consumption for complete oxidation of all substrates, 448 microliters of oxygen.

in Table II. The theoretical dilution in Experiment I was calculated by the method described by Barker and Kamen (1) and it was found that the specific activity of the succinate should have been reduced to about one-

fifth the original value if the acetate were oxidized via succinate. In Experiment II, in which the succinate was used up together with the acetate,

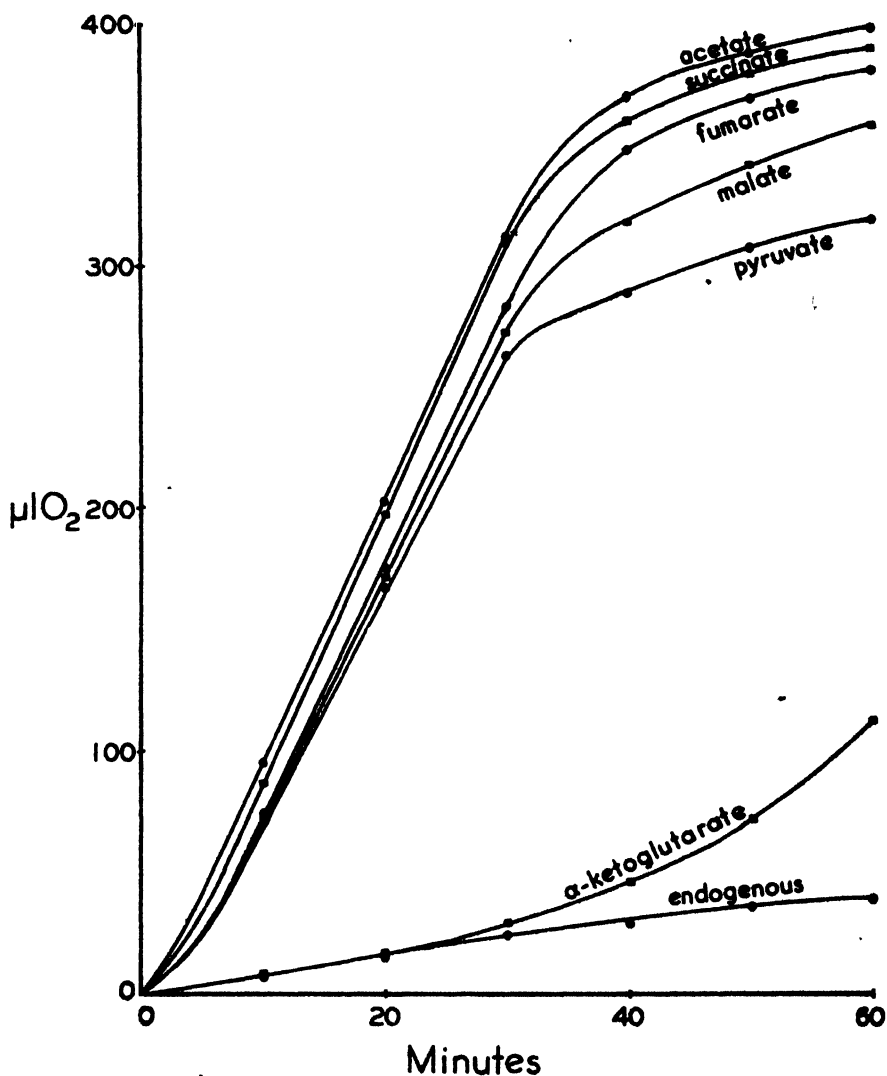


FIG. 2. Respiration curves for succinate-grown cells. Theory for complete oxidation of all substrates, 448 microliters of oxygen.

still greater dilution would be expected. The observed dilutions were of a lower order of magnitude than those expected.

Since the possibility is not excluded that the radioactive succinate does

not come into equilibrium with inactive succinate formed inside the cells, an attempt was made to demonstrate that isotopic dilution of succinate does occur during oxidation of α -ketoglutarate which in all probability is broken down via succinate. However, this experiment was not successful because a favorable ratio of the utilization of the two substrates could not be attained, since cells adapted to α -ketoglutarate attack succinate at a rapid rate.

A second experiment with C^{14} was done, this time with strain A13, an induced variant of *Azotobacter agilis* which has lost the ability to decompose oxalacetate and pyruvate (5, 6). This genetic block should not interfere

TABLE II

Test for Dilution of Radioactive Succinate during Decomposition of Inactive Acetate by Cells Unadapted to Succinate (Experiment I) and Adapted to Succinate (Experiment II)

Experiment No.		Acetic acid		Succinic acid	
		Initial	Final	Initial	Final
I	Concentration of substrates, <i>mM</i> per 100 cc.	0.333	0.030	0.167	0.125
	Specific activity, <i>counts per min. per mg.</i>			27.8	25.3
II	Concentration of substrates, <i>mM</i> per 100 cc.	1.00	0.37	0.50	
	Specific activity, <i>counts per min. per mg.</i>			27.8	24.0

TABLE III

Oxidation of Doubly Labeled Acetate in Presence of Excess Oxalacetate

			Total activity
		<i>mM</i>	<i>counts per min.</i>
Substrates	Oxalacetate	0.020	0
	Acetate	0.008	25,000
Products	β -COOH of oxalacetate	0.020	50
	CO ₂		20,000

with the occurrence of the tricarboxylic acid cycle, and the mutant oxidizes acetate at the same rate as the parent strain. The experiment was conducted in the following manner. A suspension of the organism was permitted to oxidize malate to oxalacetate, which has been shown to accumulate in the medium. Since the oxalacetate was formed inside the cells and penetrated immediately out into the medium, it seems reasonable to assume that it can also readily diffuse back in. As soon as the malate had been quantitatively converted to oxalacetate (20 minutes), a small amount of doubly labeled radioactive acetate was added. The acetate was rapidly decomposed, and according to the tricarboxylic acid cycle

theory, practically all the activity should have ended up in the oxalacetate. Such, however, was not the case, as is shown in Table III. This constitutes still another piece of evidence against the operation of the cycle in *Azotobacter agilis*.

In view of these findings, attempts were made to demonstrate the anaerobic condensation of radioactive acetate with oxalacetate or with pre-existing cell constituents, but without success. Also, possible oxidation products of acetate, such as glycolic acid, oxalic acid, and formic acid, were shown not to be attacked. Finally, the possibility of some intermediate accumulating during acetate oxidation was investigated. The organism was permitted to oxidize doubly labeled acetate until approximately 50 per cent of the theoretical amount of oxygen had been consumed. Then the reaction was stopped by addition of acid and the distribution of the radioactivity determined. The carbon dioxide was collected as BaCO_3 , and the cells were separated by centrifugation. The clear supernatant

TABLE IV
Distribution of C^{14} from Doubly Labeled Acetate after Partial Oxidation

Fraction	Total activity
	counts per min.
CO_2	8800
Cells.....	1800
Ether-insoluble compounds.....	1400
Steam-volatile acid.....	1300
Ether-soluble, non-volatile compounds.....	200

was ether-extracted overnight, and the ether extract steam-distilled. Table IV shows the distribution of the activity. No further attempts were made to identify the labeled compounds since so little C^{14} was found in the ether-soluble, non-volatile acid fraction. The ether-insoluble residue and the cells are probably too complex to make further analysis feasible.

DISCUSSION

On the basis of the data presented, it seems probable that all substrate-specific enzymes in *Azotobacter agilis* are adaptive in the sense that their activities are greatly augmented by the presence of the homologous substrates. The strongly adaptive character of the enzymes responsible for the oxidation of α -ketoglutarate, succinate, fumarate, malate, and pyruvate is clearly demonstrated in Table I and in Fig. 1. The acetate enzyme appears to be an exception, since it is present in cells grown on every substrate tested. However, this does not necessarily mean that the forma-

tion of the acetate enzyme is independent of its specific substrate. A more reasonable interpretation is that acetate is formed in the breakdown of every other substrate and so is always present to stimulate acetate enzyme formation. Independent evidence has been obtained from nutritional experiments that acetate or a closely related compound is indispensable for growth of *Azotobacter agilis* (5). It may be generally true that so called constitutive enzymes are in reality adaptive enzymes for substrates normally present in the cells as a result of endogenous reactions.

The existence of a reaction chain from α -ketoglutarate through succinate, fumarate, malate, and pyruvate to acetate is supported by the finding that each of the higher members causes adaptation to all the lower ones. But since the lower members do not cause adaptation to all the higher ones, as the tricarboxylic acid cycle theory requires, the cycle appears to be inoperative in pyruvate and acetate oxidations. Postulations of permeability phenomena being involved or modified compounds being the true intermediates are excluded by the finding that α -ketoglutarate does cause adaptation to all the lower members of the cycle. Any compound going through the same path as α -ketoglutarate after the latter has entered the main reaction chain should cause the same adaptations. The fact that this is not true for acetate can be taken as a proof that it is not metabolized via the same path as α -ketoglutarate.

The absence of the tricarboxylic acid cycle is further substantiated by the finding that even with radioactive tracer methods succinate and oxalacetate cannot be shown to be formed during acetate oxidation. Furthermore, malonate inhibition of acetate breakdown could not be demonstrated. Here again it could be maintained that the malonate does not get into the cells, particularly since the pH range is unfavorable, but even during growth on malonate, which seems to be metabolized via the same path as acetate, there is no accumulation of succinate, although in this case the malonate certainly must be getting into the cells. Finally it may be mentioned that citrate is not metabolized at all by *Azotobacter agilis*, and *cis*- and *trans*-aconitates are attacked only after long lag periods when other compounds are used as growth substrates.

Taken together, these results provide strong evidence that pyruvate and acetate are not metabolized by *Azotobacter agilis* via the generally accepted tricarboxylic acid cycle. No alternative metabolic path has been discovered, and the mechanism of acetate oxidation in this organism is still obscure.

SUMMARY

The enzymes involved in substrate oxidation in *Azotobacter agilis* are in general adaptive; *i.e.*, they are formed only when the appropriate sub-

strate is present or formed during growth. The existence of a reaction chain from α -ketoglutarate through succinate, fumarate, malate, and pyruvate to acetate is supported by the finding that each of the more reduced compounds causes adaptation to all the more oxidized ones. But since the lower compounds do not cause adaptation to the higher ones, as the tricarboxylic acid cycle theory would require, the cycle cannot be operative for pyruvate or acetate oxidation in this organism. This is also indicated by the fact that formation of succinate and oxalacetate from acetate cannot be detected even with radioactive tracers. Since acetate is oxidized at a higher rate than any other compound, a different mechanism for its breakdown must exist.

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STUDIES ON THE MECHANISM OF ALLOXAN HYPOGLYCEMIA

By SACHCHIDANANDA BANERJEE AND GANGAGOBINDA
BHATTACHARYA

(From the Departments of Chemistry and Mitra Research in Diabetes, School of Tropical
Medicine, Calcutta, India)

(Received for publication, May 22, 1948)

It is well known that when alloxan is injected intravenously into rabbits, dogs, and monkeys an initial hyperglycemia is followed by a transient hypoglycemia, leading to death of the animals from hypoglycemic convulsions. If the hypoglycemia is prevented by repeated intravenous injections of glucose, the animals survive and permanent hyperglycemia and diabetes develop.

The cause of the transient hypoglycemia has been differently explained by different workers. While Goldner and Gomori (1), Ridout *et al.* (2), Kennedy and Lukens (3), and Banerjee (4) consider that the alloxan hypoglycemia is pancreatic in origin owing to the release of preformed insulin from the necrosed islets, Houssay *et al.* (5), Wrenshall (6), and Carrasco-Formiguera (7) are of opinion that the cause of this hypoglycemia is extrapancreatic. In experiments with dogs Houssay *et al.* (5) observed hypoglycemia when alloxan was injected half an hour after the animals were pancreatectomized. Wrenshall (6) removed the pancreas of dogs 9 hours after the animals had received injections of a diabetogenic dose of alloxan and determined its insulin content. No significant difference in the insulin content as compared to normal controls was observed, which indicated that no measurable quantity of insulin was released into the circulation. Wrenshall further observed that when dogs were made resistant to insulin by injections of anterior pituitary extract a diabetogenic dose of alloxan significantly lowered the blood sugar level to that of normal controls. Carrasco-Formiguera (7) clamped the pancreaticoduodenal blood vessels in three dogs before the diabetogenic dose of alloxan was injected. Although all of the animals failed to develop diabetes, all showed pronounced hypoglycemia. In experiments with rabbits Carrasco-Formiguera observed that when rabbits received injections of epinephrine 1 hour prior to the injection of a diabetogenic dose of alloxan most of the animals failed to develop diabetes, but all of them had pronounced hypoglycemia. Goldner and Gomori (8) could not confirm the claims of Houssay and others (5-7). They observed no hypoglycemia after injection of diabetogenic doses of alloxan in dogs in which the pancreas was removed 30 minutes or several days or weeks prior to the injection. When the blood

vessels supplying the pancreas were clamped prior to the injection of alloxan, the dogs which failed to develop diabetes did not show any hypoglycemia. In their experiments with rabbits Goldner and Gomori, however, confirmed the observations of Banerjee (4) who showed that partially

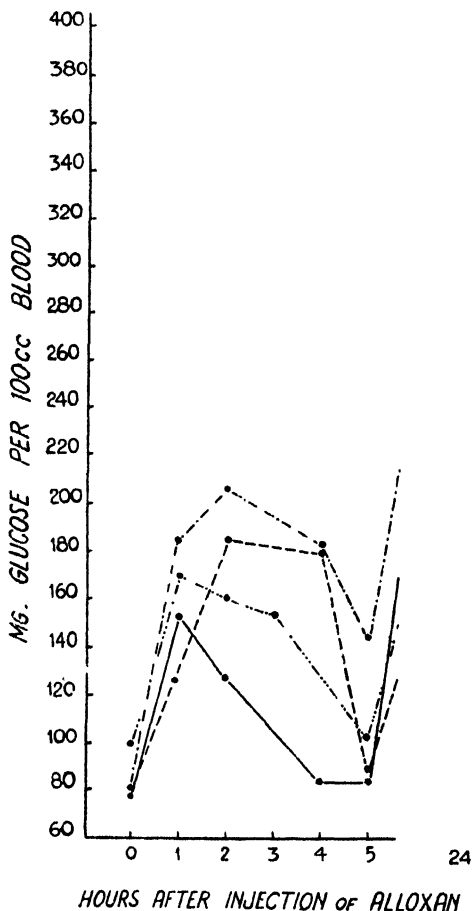


FIG. 1. Blood sugar curves of rabbits after injection of alloxan (200 mg. per kilo). Prior to the injection of alloxan the rabbits were fasted for 7 days and each received by injection a daily dose of 100 mg. of phlorhizin for 7 days.

pancreatctomized rabbits failed to develop severe hypoglycemia, did not have hypoglycemic convulsions, and all of them survived without injection of glucose and developed diabetes. These findings indicated that the hypoglycemia depended upon the amount of insulin available in the pancreas.

The present communication is intended to throw further light on the mechanism of alloxan hypoglycemia. Several rabbits were fasted for a week and injected with phlorhizin in order to lower the insulin secretion of the pancreas; the effects of the injection of diabetogenic doses of alloxan on the blood sugar level in those animals were studied.

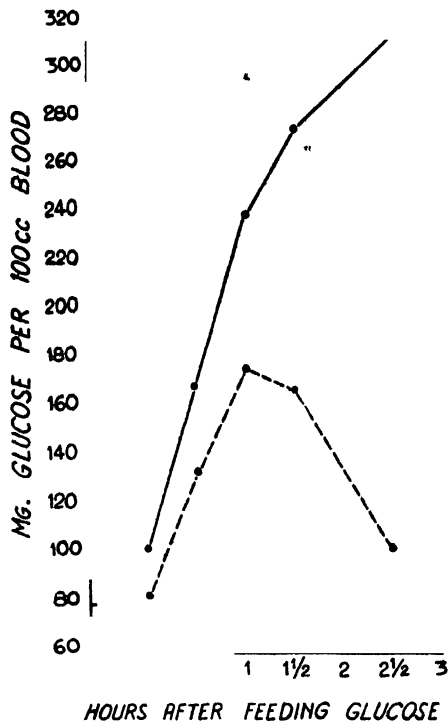


FIG. 2. Glucose tolerance curves of a rabbit. The dash curve represents the test made before the animal was fasted and phlorhizinized; the solid curve indicates the test made after the rabbit was fasted and phlorhizinized for 7 days.

EXPERIMENTAL

Four healthy female Himalayan rabbits, varying in weight between 1280 and 1530 gm., were housed in separate metabolism cages. The animals were fasted for a period of 7 days but were allowed to drink water during this period. Each animal received a daily intramuscular injection of 100 mg. of phlorhizin suspended in olive oil for these 7 days. All of the animals excreted sugar in the urine by the 2nd day of the experiment. On the 8th day alloxan in a dose of 200 mg. per kilo of body weight was injected into the marginal ear vein of all the rabbits and the blood sugar was estimated according to the method of Hagedorn and Jensen (9) in samples of blood

taken both before and at varying intervals up to 24 hours after the injection of alloxan. The blood sugar curves of the animals are shown in Fig. 1.

In order to study the utilization of glucose by a phlorhizinized rabbit fasted for 7 days, a glucose tolerance test was performed in a rabbit fasted overnight. The rabbit was fasted and then given a daily injection of 100 mg. of phlorhizin for a period of 7 days as described before. On the 8th day the glucose tolerance test was performed as follows: The rabbit was fed a 50 per cent solution of glucose in a dose of 1 gm. per kilo of body weight. Samples of blood were taken both before and at intervals of half an hour up to 2½ hours after the glucose feeding. Blood sugar was determined as before. The results are shown in Fig. 2.

Two rabbits were made diabetic by intravenous injection of alloxan. After 7 days a diabetic type of glucose tolerance curve was obtained.

TABLE I

Blood Sugar Values of Alloxan-Diabetic Rabbits before and after Intravenous Injection of Alloxan

Blood sugar values are expressed in mg. per cent.

Weight of rabbit <i>gm.</i>	Fasting blood sugar	Blood sugar after injection of alloxan					
		½ hr.	1½ hrs.	2½ hrs.	3½ hrs.	4½ hrs.	6 hrs.
1490	233	512	497	428	428	428	462
1500	118	204	240	287	341	259	163

Alloxan (200 mg. per kilo) was then injected in both of these rabbits and samples of blood were drawn at varying intervals up to 6 hours after the injection of alloxan. The blood sugar values are given in Table I.

Results

Phlorhizinized rabbits fasted for 7 days showed a diabetic type of glucose tolerance curve. Injection of alloxan into such rabbits produced an initial hyperglycemia, but no hypoglycemia was observed in any one of them. 24 hours after the injection of alloxan all of the rabbits excreted sugar in the urine and the blood sugar level was high, indicating that all of the animals developed diabetes.

Injection of alloxan into rabbits made diabetic by a previous injection of alloxan caused a further rise in the blood sugar level, and even 6 hours after the injection of alloxan the blood sugar level was much above the fasting blood sugar value.

DISCUSSION

Normal rabbits develop hypoglycemic convulsions within 2 to 4 hours after the intravenous injection of a diabetogenic dose of alloxan (200 mg. per kilo) (4). All of the four rabbits which were phlorhizinized and fasted for a period of 7 days showed initial hyperglycemia, contrary to the findings of Goldner and Gomori (10), but failed to develop hypoglycemia even 5 hours after the injection of alloxan. All of the animals survived the next day without injection of glucose and showed marked hyperglycemia and glycosuria and developed diabetes. A glucose tolerance test in one such rabbit gave a diabetic type of curve, indicating that a fasted phlorhizinized rabbit has possibly less insulin for the utilization of glucose. Du Vigneaud and Karr (11) observed that rabbits fasted for 7 days or longer did not utilize glucose as normal animals do. The injection of phlorhizin to the fasted rabbit led to the excretion of glucose in the urine, which further enhanced the carbohydrate starvation, leading possibly to further diminution in the insulin secretion of the pancreas. Injection of alloxan in two alloxan-diabetic rabbits failed to lower the blood sugar level from the initial fasting value. The absence of hypoglycemia after the injection of a diabetogenic dose of alloxan in fasted and phlorhizinized rabbits and in rabbits made diabetic by previous injection of alloxan, conditions in which there is less insulin in the pancreas, seems to indicate that the cause of alloxan hypoglycemia is pancreatic in origin and not extrapancreatic as claimed by Houssay and others (5-7).

SUMMARY

1. Alloxan (200 mg. per kilo) was injected intravenously into four rabbits which were fasted and then received a daily injection of 100 mg. of phlorhizin for a period of 7 days. No hypoglycemia was observed in any of the rabbits.

2. Glucose tolerance tests were performed in a rabbit, both before and after the animal was phlorhizinized and fasted for 7 days. A diabetic type of glucose tolerance curve was observed when the animal was phlorhizinized and fasted.

3. Alloxan was injected into two rabbits made diabetic by a previous injection of alloxan. Alloxan did not lower the blood sugar level from its initial fasting value.

4. The alloxan hypoglycemia as observed in rabbits seems to be pancreatic in origin.

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THE STRUCTURE OF A NEW CYCLOHEXOSE PRODUCED FROM *d*-INOSITOL BY BIOLOGICAL OXIDATION*

By BORIS MAGASANIK† AND ERWIN CHARGAFF

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, May 25, 1948)

In a previous publication (1) the oxidation products were described that resulted from the action of *Acetobacter suboxydans* on various isomers of the inositol series. With *d*-inositol (I) as the substrate, the bisphenylhydrazone of the diketone II and a compound having the analytical composition of the phenylhydrazone of a cyclohexose were isolated after the addition of phenylhydrazine to the culture fluid. Both substances were obtained in very poor yields. Since, however, *d*-inositol was shown to take up 2 gm. atoms of oxygen per mole, when shaken in a Warburg vessel with resting bacteria, an extension of this technique to preparative work seemed promising. The results of these experiments, which led to the isolation of a new cyclohexose, are described in the present paper.

The apparatus used, essentially identical with the customary arrangement for catalytic hydrogenations at atmospheric pressure, made it possible, by means of the observation of oxygen consumption, to follow the course of the reaction. The oxidative reactions could be interrupted at various levels of oxygen uptake, yielding different proportions of the mono- and the diketo compound. The addition of phenylhydrazine to the solutions containing these substances resulted in the precipitation of mixtures of their respective mono- and bisphenylhydrazones. These could be separated by virtue of the lower solubility of the monophenylhydrazone in boiling ethanol. The pure compound, however, was not obtained in a good yield when large amounts of the bisphenylhydrazone were present. For the preparation of the monoketo compound the reaction was, therefore, interrupted after the uptake of about 0.5 gm. atom of oxygen per mole of *d*-inositol. Under these conditions the yield of monophenylhydrazone was 55 per cent and of bisphenylhydrazone 3.3 per cent of the theoretical, based on the amount of oxygen consumed.

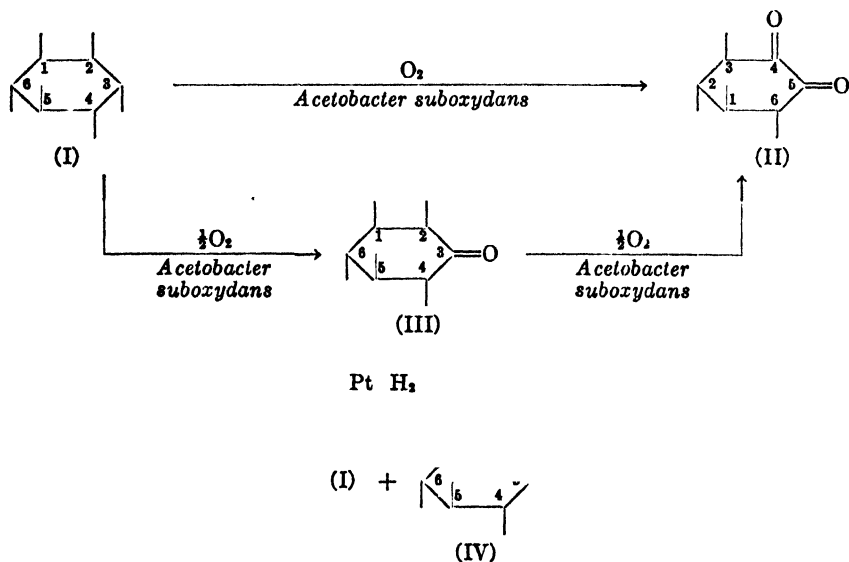
The levorotatory phenylhydrazone was split in the usual manner with

* This work was supported in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

† William J. Gies Fellow, 1947-48. This report is from a dissertation submitted by Boris Magasanik in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

benzaldehyde. The resulting inosose was found to be less stable than the inososes previously described (2-5). The evaporation of the solution containing this compound to dryness *in vacuo* at about 30° led to a non-crystalline residue which failed to reduce Benedict's solution. When, however, the evaporation was carried out in the frozen state *in vacuo*, the hemihydrate of an inosose, $C_6H_{10}O_6 \cdot \frac{1}{2}H_2O$, was obtained, after the recrystallization of the residue from aqueous ethanol, in a yield of 87 per cent of the theory. The dextrorotatory, very water-soluble, crystalline substance reduced Benedict's solution in the cold, though more slowly than *meso*- or *epi*-inosose, and reacted with phenylhydrazine to form a phenylhydrazone.

The exact configuration (III) of this cyclohexose, for which the designation *d*-inosose is proposed, was established by oxidation with resting *Acetobacter suboxydans*, which led, after uptake of 1 gm. atom of oxygen per mole of substrate, to a diketone isolated as its bisphenylhydrazone. This was shown by optical rotation, melting point, and mixed melting point to be identical with the compound resulting from the uninterrupted bacterial oxidation of *d*-inositol (I) itself (1). The reactions involved are represented in the accompanying flow sheet. (The numbering of the carbon atoms follows that adopted in the preceding paper (1).)



Since II is formed by the oxidation of the hydroxyl groups in positions 2 and 3 of I (1), *d*-inosose must be the monocarbonyl compound obtained by the oxidation of one of these groups. The inspection of the structural formula shows that, regardless which of these hydroxyls is attacked, cyclohexane-(1,2,5)*cis*-pentol-3-one (III) will result.

Additional proof was furnished by the reduction of *d*-inosose with hydrogen in the presence of platinum oxide. This reaction led to a mixture of the stereoisomers *d*-inositol (I) and *meso*-inositol (IV), in which compound I predominated. This is an instance of the conversion of one naturally occurring isomer, *viz.* *d*-inositol, into another, *meso*-inositol.

d-Inosose (III) belongs to the group of six cyclohexoses structurally derived from *meso*-inositol (IV). Of these, *meso*-inosose (3), *dl*-*epi*-inosose (2), and *l*-*epi*-inosose (4, 5), which have already been described, closely resemble each other in chemical and physical properties. *d*-Inosose, however, differs markedly from the others. Reference has already been made to its greater heat sensitivity, to its slower oxidation by Benedict's solution, and to the fact that it crystallized from aqueous alcohol as a stable hemihydrate.

Striking differences were revealed by a comparison of the ultraviolet absorption spectra of *meso*- and *d*-inosose (Fig. 1). *Meso*-Inosose, in a freshly prepared aqueous solution, was found to possess an absorption band with a maximum at $282\text{ m}\mu$ ($\epsilon = 22.2$), in essential agreement with the observation of Posternak (5). The same spectrum was recorded when the measurement was repeated after the solution had been allowed to stand at room temperature for 48 hours. A freshly prepared aqueous solution of *d*-inosose, on the other hand, showed a weak absorption band with a maximum at $258\text{ m}\mu$ ($\epsilon = 14.8$). But the spectrum changed, in the beginning quite rapidly, when the solution was stored, with the disappearance of the absorption band. The curve recorded after 48 hours is included in Fig. 1. Absorption bands in this region of the spectrum are characteristic for the carbonyl group (6). According to this evidence, *meso*- and *epi*-inosose, as was pointed out by Posternak (5), seem to exist in aqueous solution as the free keto compounds, differing in this respect from the ordinary hexoses. *d*-Inosose, on the other hand, does not appear to occur to any extent as a carbonyl compound, once tautomeric equilibrium has been established.

Another unusual feature may be seen in the behavior of *d*-inosose toward sodium amalgam. Whereas the other inososes studied were reduced by this reagent to a mixture of stereoisomeric inositols, *d*-inosose yielded, following the acetylation of the reduction product, an unexpected derivative. The analytical figures obtained for this crystalline compound and its molecular weight were in keeping with its formulation as the decaacetate of a substance having the formula $\text{C}_{12}\text{H}_{12}\text{O}(\text{OH})_{10}$ (V) or $\text{C}_{12}\text{H}_{10}\text{O}(\text{OH})_{10}$ (VI). In V, 2 molecules of inositol could be assumed to be connected through an ether linkage $>\text{CH}-\text{O}-\text{CH}<$; VI, containing 2 hydrogen atoms less,

could be formulated as an epoxide, $>\text{C} \begin{array}{c} \text{O} \\ \diagup \quad \diagdown \\ \text{---} \end{array} \text{C}<$.

If *d*-inosose itself is considered as existing largely as the dimer, in which the 2 molecules are linked by way of the hydrated carbonyl groups, $>\text{C}(\text{OH})-\text{O}-\text{C}(\text{OH})<$, the analytically demonstrated presence of 1 molecule of stably bound water for 2 molecules of the keto compound becomes understandable. The other chemical and physical properties of the compound, mentioned above, also accord with this assumption, although the

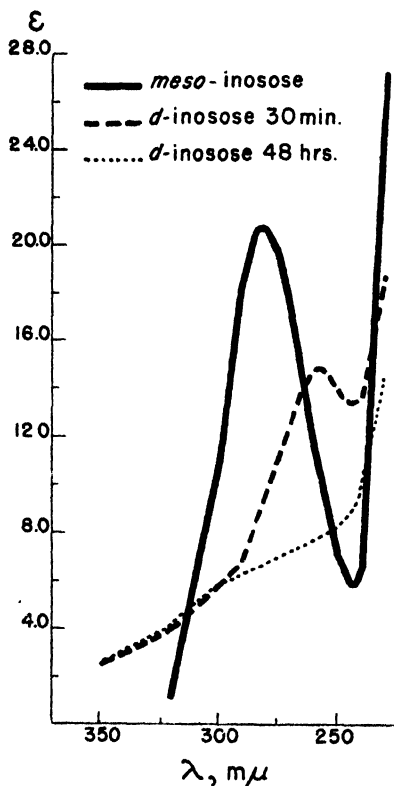


FIG. 1. Absorption spectra of *d*-inosose (III) and *meso*-inosose, 30 minutes and 48 hours after the preparation of the aqueous solutions.

production of an ether linkage (V) by the reduction of a hemiacetal with sodium amalgam appears rather unusual.

In the second, less plausible alternative mentioned above, the decaacetate of compound VI would have been formed from monomeric *d*-inosose by the reduction of 2 molecules to a pinacol which then was dehydrated, under the conditions of the acetylation, to an epoxide. While analogous reactions have been described (7, 8), it is difficult to correlate the properties of *d*-inosose with this assumption.

EXPERIMENTAL

Material

The preparation of *d*-inositol and the cultivation of *Acetobacter suboxydans* have been described in a previous communication (1).

Preparation of d-Inosose

18 gm. (100 mm) of *d*-inositol were dissolved in a mixture of 100 cc. of water and 50 cc. of M/15 phosphate buffer of pH 6.0 in an Erlenmeyer flask. To this solution a suspension of washed *Acetobacter suboxydans*, grown in nine Roux bottles for 3 days (about 0.5 gm., dry weight), in 25 cc. of 0.9 per cent aqueous sodium chloride was added. The flask was attached to a graduated reservoir filled with oxygen and the mixture agitated at 37° with a magnetic stirrer. The rapid reaction was stopped after 155 minutes, when 565 cc. of oxygen (about 23 mm) had been taken up. The bacteria were removed by centrifugation and filtration through infusorial earth, and 10 cc. of phenylhydrazine in 20 cc. of 50 per cent acetic acid were added to the clear, colorless filtrate. The solution turned red, and a copious precipitation of pink crystals occurred. This mixture of phenylhydrazones was washed with water, dried in air, and the dry material (9.8 gm.) was refluxed for 30 minutes with 200 cc. of ethanol. The mixture was filtered and the yellowish residue again extracted under a reflux with 200 cc. of ethanol for 18 hours. The undissolved residue consisted of 6.8 gm. (25.4 mm) of almost pure *phenylhydrazone of d-inosose*, m.p. 193–195°.¹ Yield, 55 per cent, based on the amount of oxygen consumed. The preparation of the pure phenylhydrazone is described below.

The red alcoholic filtrates, on evaporation to a small volume and addition of water, yielded 270 mg. (0.76 mm) of the previously described (1) *bis-phenylhydrazone of II*, m.p. (after recrystallization from aqueous methyl cellosolve) 217°. Yield, 3.3 per cent, based on the amount of oxygen consumed.

The phenylhydrazone (6.8 gm.) was heated under a reflux for 10 minutes with 10 cc. of freshly distilled benzaldehyde and 1 gm. of benzoic acid in 100 cc. of water. The filtrate was extracted with three 100 cc. portions of ether and treated with charcoal, and the clear, yellow solution evaporated *in vacuo* in the frozen state. The yellowish, semicrystalline residue was dissolved in 20 cc. of water, containing a few drops of dilute sulfuric acid, and decolorized with charcoal, and 60 cc. of ethanol were added to the clear, colorless filtrate. After chilling overnight, the crystalline hemihydrate of

¹ The melting points, reported without correction, were determined with an electrically heated stage (Fisher-Johns). The intact crystals were placed on the stage which was preheated to about 5–7° below the melting point.

d-inosose (III) was obtained as colorless plates weighing 4.1 gm. (22 mm). Yield, 87 per cent of the theoretical.

After one additional crystallization from aqueous ethanol, the compound melted at 138–139°. It was dextrorotatory in water and showed no mutarotation ($c = 2.37$, $l = 2$ dm., $\alpha_D^{25} = +0.93^\circ \pm 0.01^\circ$); $[\alpha]_D^{25} = +19.6^\circ \pm 0.2^\circ$. Its solution reduced Benedict's solution in the cold, though at an appreciably slower rate than a corresponding solution of *meso*-inosose.

$C_6H_{10}O_6 \cdot \frac{1}{2}H_2O$ (187.1). Calculated, C 38.5, H 5.9; found, C 38.5, H 5.8

Phenylhydrazone of d-Inosose—On addition of phenylhydrazine in 50 per cent acetic acid to an aqueous solution of *d*-inosose, faintly pink crystals appeared almost at once. They were washed with hot ethanol and recrystallized from aqueous pyridine. The *phenylhydrazone of III* was thus obtained as white plates, melting with decomposition at 196–197°. The optical rotation in 1:1 ethanol-pyridine ($c = 1.80$, $l = 2$ dm., $\alpha_D^{25} = -1.99^\circ \pm 0.02^\circ$) was $[\alpha]_D^{25} = -55.3^\circ \pm 0.5^\circ$. No mutarotation was observed within 24 hours.

$C_{12}H_{16}O_8N_2$ (268.3). Calculated. C 53.7, H 6.0, N 10.4
Found. " 53.8, " 5.7, " 10.5

No crystalline acetate of *d*-inosose could be obtained.

Action of Acetobacter suboxydans on d-Inosose (III)

Oxygen Consumption and Oxidation Rate—The determinations were carried out by allowing 0.5 cc. of a bacterial suspension containing 1.3 mg. of bacterial nitrogen to act on 7 micromoles of *d*-inosose in a total volume of 3 cc. of M/15 phosphate buffer of pH 6.0 and at 36° in the presence of air. The uptake of oxygen per mole of substrate was 0.51 mole. The Q_{O_2} (N) calculated for the time interval for which the oxygen uptake was linear was –83. The total duration of oxygen uptake was about 80 minutes.

Formation of Bisphenylhydrazone of II—In 3 cc. of M/15 phosphate buffer (pH 6.0), 187 mg. (1 mm) of III were dissolved and shaken under oxygen with a suspension of washed *Acetobacter suboxydans* (dry weight about 0.2 gm.) in a water bath maintained at 36°. A total of 12 cc. of oxygen (about 0.50 mm) was taken up in the course of 10 hours. The bacteria were removed by centrifugation and filtration through infusorial earth, and 1.0 cc. of phenylhydrazine in 2.0 cc. of 50 per cent acetic acid was added to the clear, yellowish filtrate. A reddish yellow precipitate appeared, which yielded, after recrystallization from aqueous ethanol, 140 mg. (0.39 mm, 39 per cent of the theoretical) of the *bisphenylhydrazone of II*. This compound, whose direct preparation from *d*-inositol has already been described (1), was further purified by recrystallization from aqueous methyl cellosolve. The long yellow crystals, possessing a greenish sheen, melted at 217° with

decomposition. The crushed powder decomposed at 210° (1). The optical rotation of the freshly prepared solution of the compound in 1:1 ethanol-pyridine ($c = 0.056$, $l = 1$ dm., $\alpha_D^{25} = -0.16^{\circ} \pm 0.01^{\circ}$) was $[\alpha]_D^{25} = -286^{\circ} \pm 17^{\circ}$. The bisphenylhydrazone, obtained by the direct bacterial oxidation of *d*-inositol (1), showed an optical rotation of ($c = 0.0504$, $l = 1$ dm., $\alpha_D^{25} = -0.14^{\circ} \pm 0.01^{\circ}$) $[\alpha]_D^{25} = -278^{\circ} \pm 20^{\circ}$.² The mixture of these two bisphenylhydrazones showed no depression of the melting point.

Conversion of d-Inosose to d- and meso-Inositol

Catalytic Reduction—1 gm. (5.4 mm) of III was dissolved in 30 cc. of water and shaken with 440 mg. of platinum oxide in an atmosphere of hydrogen. A total of 225 cc. of hydrogen was taken up in 3 hours. About 1 mole of hydrogen was consumed per mole of *d*-inosose (after correction for the hydrogen used for the reduction of the catalyst). A little acetic acid was added, the mixture heated to boiling, the coagulated platinum removed by filtration, and the filtrate evaporated to dryness *in vacuo*. The residue was dissolved in 5 cc. of water and the solution treated with charcoal. The addition of 40 cc. of ethanol to the filtrate resulted in the deposition of a mixture of *meso*- and *d*-inositol, 620 mg. (64 per cent of the theoretical) melting, after being dried *in vacuo* at 60° , between 223 – 236° .

Hexaacetate of meso-Inositol—A portion of the mixture of the two inositols (85 mg.) was refluxed for 8 minutes with 1 cc. of acetic anhydride in the presence of a small amount of zinc chloride. The cooled mixture was poured into 10 cc. of water, when a viscous oil, adhering to the sides of the flask, separated. The supernatant liquid was decanted from the chilled mixture and the oil dissolved in a small volume of boiling ethanol. The concentration of this solution to a volume of 1 cc., followed by cooling, resulted in the deposition of about 10 mg. of white crystals. Their melting point, after one additional recrystallization from ethanol, was 216 – 217° . The admixture of authentic *hexaacetyl meso-inositol* did not lower the melting point.

The alcoholic mother liquors were evaporated to dryness and the resulting viscous oil, presumably *d-inositol hexaacetate*, was found to weigh 65 mg. after being dried *in vacuo* at 60° .

***d*-Inositol**—The remainder of the mixture of the two inositols was re-

² Higher values for the specific rotation were observed in more concentrated solutions of the bisphenylhydrazones (1). In the dilute solutions used in the experiments here described, the rotation changed very rapidly. After 24 hours the solution of the bisphenylhydrazone of II was found to have become dextrorotatory, and that of its enantiomorph (1) levorotatory. No such rapid changes were observed in the more concentrated solutions (1). This is in keeping with the findings of Engel (9) who showed the mutarotation of osazones to be due to hydrolysis to the free diketone and phenylhydrazine.

crystallized twice from aqueous alcohol. The white crystals (130 mg.), melting at 247–248°, showed no depression of the melting point when mixed with *d-inositol*. The optical rotation in water ($c = 3.30$, $l = 2$ dm., $\alpha_D^{25} = +4.20^\circ \pm 0.02^\circ$) was found as $[\alpha]_D^{25} = +63.6^\circ \pm 0.3^\circ$. The rotation of pure *d-inositol* is $[\alpha]_D^{25} = +65.0^\circ$.

Reduction of *d-Inosose* (III) by Sodium Amalgam

To 700 mg. (3.8 mm) of III, dissolved in 10 cc. of water, 2.5 per cent sodium amalgam was added in 4 gm. portions over a period of several hours with continuous shaking. The solution was kept neutral by the addition of *N* acetic acid. After 40 gm. of sodium amalgam had been added, the mixture no longer reduced Benedict's solution. The mercury was removed by filtration and the filtrate evaporated to dryness *in vacuo*. The dried residue was treated with 10 cc. of boiling acetic anhydride in the presence of zinc chloride for a period of 5 minutes. The cooled mixture was poured into several volumes of water and chilled overnight, when 620 mg. of slightly brown, crystalline material, melting at about 170°, were deposited. The filtered solution of this substance in boiling ethanol deposited, on being cooled, 450 mg. of white plates, melting at 175–176°. This compound did not reduce Benedict's solution, even after prolonged heating.

Decaacetate of $C_{12}H_{18}O(OH)_{10}$, $C_{22}H_{40}O_{21}$

Calculated. C 50.4, H 5.6, CH_3CO — 56.4, mol. wt. 762.7

Decaacetate of $C_{12}H_{10}O(OH)_{10}$, $C_{12}H_{40}O_{21}$

Calculated. C 50.5, H 5.3, CH_3CO — 56.6, mol. wt. 760.6

Found. " 50.6, " 5.5, " 56.0, " " (Rast) 730

Absorption Spectra

The absorption spectra of *d-inosose* (III) (24.4 mm solution in water) and of *meso-inosose* (28.9 mm solution in water), 0.5 and 48 hours after preparation of the solutions, are reproduced in Fig. 1. The pH of the solution was 5.4 and 4.8 respectively. The measurement of the spectra in solutions adjusted to pH 7.0 by dilute phosphate buffer led to essentially the same results. A Beckman photoelectric quartz spectrophotometer was used.

We are indebted to Miss R. Rother for the microanalyses. The acetyl determination was carried out through the courtesy of Dr. Al Steyermark of Hoffmann-La Roche, Inc., Nutley, New Jersey.

SUMMARY

A new cyclohexose, *d-inosose* (III), was obtained by the incomplete oxidation of *d-inositol* (I) by *Acetobacter suboxydans*.

The configuration of this compound, which appears to exist as the dimer, was established by bacterial oxidation to the diketone II and by catalytic reduction to *d*-inositol (I) and *meso*-inositol (IV).

In contrast to the other known inososes, *d*-inosose exhibited, in a freshly prepared aqueous solution, only a weak carbonyl band in the ultraviolet, which disappeared when the solution aged.

The reduction of the keto compound with sodium amalgam led to a dimeric compound, isolated as the decaacetate, whose structure is discussed.

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THE OXIDATION OF *d*-QUERCITOL BY ACETOBACTER SUBOXYDANS*

By BORIS MAGASANIK† AND ERWIN CHARGAFF

(From the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, May 25, 1948)

A previous communication from this laboratory (1) included the demonstration that *d*-quercitol (I) was oxidized by resting *Acetobacter suboxydans*. The maximal oxygen consumption amounted to 2 gm. atoms per mole of substrate. The present paper deals with the isolation and identification of the oxidation product.



(I)

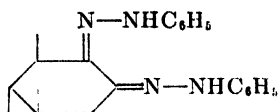
The oxidation was carried out on a preparative scale with resting bacteria in the presence of oxygen by the technique described in the preceding paper (2). Oxygen uptake under these conditions ceased when about 1.3 atoms of oxygen had been taken up per molecule of *d*-quercitol. The addition of phenylhydrazine to the reaction mixture resulted in the deposition of a dextrorotatory, crystalline, yellow compound, having the analytical composition of the bisphenylhydrazone of a diketo quercitol. The yield was 33 per cent of the theoretical, based on the amount of oxygen consumed. The low recovery of bisphenylhydrazone and the incomplete oxidation (measured by the oxygen uptake) seem to indicate that the attack on a large portion of the substrate stopped with the formation of an intermediate monoketo compound. This finding is in keeping with observations reported previously (1), which showed that the rate of the first oxidation step was considerably greater than that of the second, and that with a smaller quantity of bacteria the total oxygen uptake amounted to only 1 atom per molecule of substrate. Attempts at the isolation from the reaction mixture of the phenylhydrazone of a monoketo compound were, however, not successful.

The absorption spectrum of the bisphenylhydrazone reproduced in Fig. 1

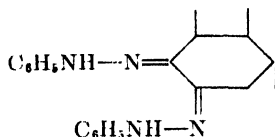
* This work was supported in part by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

† William J. Gies Fellow, 1947-48. This report is from a dissertation submitted by Boris Magasanik in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Pure Science, Columbia University.

is characteristic of osazones (1, 3). The consumption of periodic acid in 66 per cent ethyl alcohol at room temperature corresponded to 2 moles of oxidant per mole of substance. The compound must therefore be the bis-phenylhydrazone of an α -diketone, with the three hydroxyl groups situated on adjacent carbon atoms. Structures II and III only are compatible with these results.

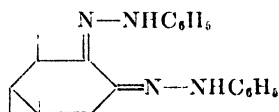


(II)



(III)

In order to decide between these two possible structures, the rates of oxidation by periodic acid of this α -bisphenylhydrazone and of the one prepared from *D*-inositol (IV) (1) were compared.



The measurements were carried out in very dilute, strongly acidic, alcoholic solution, in order to allow observation by slowing down the reaction rates sufficiently. The results summarized in Table I reveal that the α -bisphenylhydrazone derived from *D*-quercitol was oxidized in the early phase of the reaction at a speed about half of that at which the *D*-inositol derivative was attacked.

It has been shown repeatedly (4, 5) that cyclohexitols possessing two vicinal hydroxyl groups in the *cis* position are cleaved by periodic acid at an appreciably greater rate than are their *trans* isomers.

Compound IV (1) does not possess a pair of vicinal *cis* hydroxyls. A compound having structure III, with one pair of *cis* hydroxyls, may therefore be expected to be oxidized more rapidly than IV. The observation that the α -bisphenylhydrazone derived from *D*-quercitol was oxidized at a considerably slower rate than IV favors the assumption that it possesses structure II, in which the vicinal hydroxyl groups are in the *trans* position. The slower oxidation may be due to the fact that compound II has only three hydroxyl groups, whereas compound IV has four.

The evidence presented indicates that the hydroxyl groups in positions 2 and 3 of *D*-quercitol (I) are oxidized by *Acetobacter suboxydans* to give

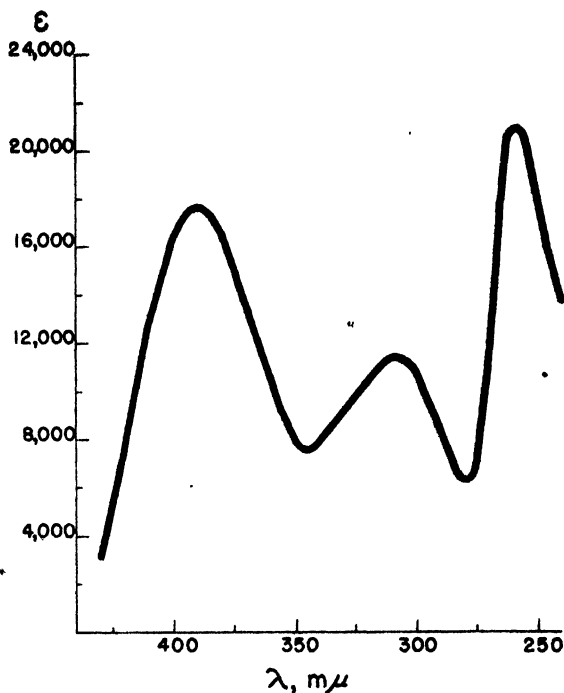


FIG. 1. Absorption spectrum (in absolute ethanol) of the α -bisphenylhydrazone of cyclohexane-(1,5)*cis*-6-triol-2,3-dione (II).

TABLE I

Rates of Oxidation by Periodic Acid of α -Bisphenylhydrazones Derived from d-Quercitol and d-Inositol

Duration of oxidation	Oxidant consumed per mole α -bisphenylhydrazone derived from	
	d-Quercitol	d-Inositol
min.	moles	moles
1	0.30	0.55
2	0.40	0.86
5	0.68	1.25
10	0.98	1.48
30	1.40	1.78
1440	2.35	3.01

cyclohexane-(1,5)cis-6-triol-2,3-dione, isolated as its bisphenylhydrazone (II).¹

¹ With respect to the numbering of the carbon atoms, compare a previous discussion (1).

The steric structure of *d*-quercitol has been discussed in a previous communication (1). It was suggested there that the hydroxyl groups 2 and 3 are situated in the north and south polar planes, respectively, while the remaining hydroxyls are ranged in the equatorial belt. The results reported in this paper are, therefore, in agreement with the previously stated rule for the minimum steric requirements for the oxidation of inositol isomers by *Acetobacter suboxydans*; viz., that only polar hydroxyl groups are oxidized (1).

It may be of interest to point out that Bertrand's rule (6), which describes the action of the microorganism on straight chain polyhydroxy compounds, would have led to the expectation that a monoketo compound should be formed from *d*-quercitol.

EXPERIMENTAL

Material

d-Quercitol was a commercial preparation, melting at 239–240°, $[\alpha]_D^{23} = +23.9^\circ$. *Acetobacter suboxydans* was cultivated by the methods described previously (1).

Formation of Compound II from d-Quercitol

To a solution of 1.76 gm. (10.7 mm) of *d*-quercitol in 25 cc. of M/15 phosphate buffer of pH 6.0 a suspension of washed *Acetobacter suboxydans* (about 0.5 gm., dry weight) in 25 cc. of 0.9 per cent aqueous sodium chloride was added. The flask was attached to a graduated reservoir filled with oxygen and the mixture agitated with a magnetic stirrer in a room maintained at 37°. The consumption of oxygen, rapid at first, ceased when 167 cc. (about 6.7 mm) of oxygen had been taken up in 230 minutes. The bacteria were removed by centrifugation and filtration through infusorial earth and 4 cc. of phenylhydrazine in 8 cc. of 50 per cent acetic acid were added to the clear, yellow filtrate. The precipitate (1.45 gm.) weighed, after being washed with cold ethanol, 750 mg. (2.2 mm, 33 per cent of the theoretical yield, based on the amount of oxygen consumed) and formed pale yellow crystals. The *bisphenylhydrazone* of cyclohexane-(1,5)*cis*-6-*triol*-2,3-*dione* (II), after two recrystallizations from aqueous methyl cellosolve and boiling ethanol, melted (with decomposition) at 199–200°. The optical rotation in 1:1 ethanol-pyridine ($c = 0.357$, $l = 0.5$ dm., $[\alpha]_D^{25} = +0.11^\circ$) was found as $[\alpha]_D^{25} = +62^\circ$.

$C_{18}H_{28}O_8N_4$ (340.4). Calculated. C 63.5, H 5.9, N 16.5

Found. " 63.1, " 5.6, " (Dumas) 16.6

* The melting points, reported without correction, were determined with an electrically heated stage (Fisher-Johns). The intact crystals were placed on the stage which was preheated to about 5–7° below the melting point.

The various mother liquors yielded, on evaporation, red amorphous powders which were very soluble in alcohol.

Absorption Spectrum—The absorption spectrum of II (0.0288 mm solution in absolute ethanol) is reproduced in Fig. 1. The band at $258\text{ m}\mu$ ($\epsilon = 20,900$) has the same center of absorption as the corresponding bands of the bisphenylhydrazones derived from *l*- and *d*-inositol (1). The other two bands of II at $308\text{ m}\mu$ ($\epsilon = 11,400$) and $389\text{ m}\mu$ ($\epsilon = 17,700$) are at slightly lower wave-lengths than the corresponding bands of the inositol derivatives.

Action of Periodic Acid on Compound II

Total Consumption—The HIO_4 consumption was determined by treating 20 cc. portions (each containing about 60 micromoles of the bisphenylhydrazone in 60 per cent ethanol) with 234 micromoles of periodic acid in 0.5 cc. of water at room temperature for 1 hour (1, 7). The excess periodic acid was determined with 0.1 N sodium arsenite in the usual manner. The average consumption (per mole of compound) of periodic acid was 2.1 moles.

Rate of Periodic Acid Oxidation of Compounds II and IV—To 10 cc. of an aqueous solution containing 2.18 micromoles of periodic acid and 0.5 cc. of 4 N sulfuric acid, 0.5 micromole of the bisphenylhydrazones in 0.5 cc. of ethanol was added. The reaction was stopped by the addition of 2 cc. of a saturated solution of potassium bicarbonate and some solid potassium iodide. The excess periodic acid was determined in the customary manner with 0.01 N sodium arsenite with the aid of a micrometric burette (8). The results are summarized in Table I.

We are indebted to Miss R. Rother for the microanalyses.

SUMMARY

Following the oxidation of *d*-quercitol (I) by resting *Acetobacter suboxydans*, the α -bisphenylhydrazone (II) of a diketo quercitol was isolated and its structure determined. The results show that hydroxyl groups 2 and 3 of *d*-quercitol, which are situated in polar planes, were oxidized. This is in agreement with the previously stated rule for the minimum steric requirements for the oxidation of inositols by *Acetobacter suboxydans*.

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EXPERIMENTALLY INDUCED CHANGES IN THE PROTHROMBIN LEVEL OF THE BLOOD

IV. THE RELATION OF VITAMIN K DEFICIENCY TO THE INTENSITY OF DICUMAROL ACTION AND TO THE EFFECT OF EXCESS VITAMIN A INTAKE; WITH A SIMPLIFIED METHOD FOR VITAMIN K ASSAY*

BY ARMAND J. QUICK AND MARIO STEFANINI†

*(From the Department of Biochemistry, Marquette University School of
Medicine, Milwaukee)*

(Received for publication, June 11, 1948) .

Many problems concerning prothrombin and vitamin K require an accurate but relatively simple method for assaying the latter agent. Most of the early methods were crude, since they were based on the coagulation time which is only roughly related to the prothrombin concentration (1). Schoenheyder's method (2), published in 1936, was an exception, since he determined the prothrombin time indirectly by comparing the amount of thromboplastin that had to be added to normal and to avitaminotic plasma to produce the same coagulation time. The method is empirical and subject to serious errors, since it does not specifically measure prothrombin. Nevertheless, it has in the hands of Dam and his associates yielded much valuable information.

Independently, Quick (3) devised an assay method for vitamin K using as the basis his prothrombin time procedure. He showed that 1 per cent alfalfa meal added to Almquist's diet (4) was enough to protect chicks from hemorrhage, but 2 per cent was required to restore completely the prothrombin level. Almquist and Klose (5) later adopted the prothrombin time in their assay procedure but unfortunately selected as their thromboplastin chicken breast muscle which has a relatively low activity and contains clotting factors other than thromboplastin. Stamler, Tidrick, and Warner (6) more recently have outlined a procedure which is highly sensitive and accurate, but has the disadvantage of depending on the two-stage prothrombin determination which is time-consuming and technically difficult.

To meet the needs for a practical and satisfactory method of assaying vitamin K, a study was undertaken to devise a diet consisting of constituents readily available commercially, which would obviate the need of

* This work was supported by a grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

† Department of Internal Medicine, University of Rome; at present, Senior Research Fellow, National Institute of Health, United States Public Health Service.

long laborious extraction, and to outline a procedure based on the one-stage prothrombin method. With the successful accomplishment of this task, several problems were studied: (1) the relative potency of menadione (2-methyl-1,4-naphthoquinone) and natural vitamin K₁, (2) the speed with which prothrombin activity is restored by vitamin K, (3) the influence of vitamin K on the effectiveness of dicumarol, and (4) the effect of high vitamin A intake on the prothrombin level when a minimal maintenance dose of vitamin K is given.

EXPERIMENTAL

The diet which was found completely satisfactory, since it produced a high degree of hypoprothrombinemia in 10 days yet maintained satisfactory growth, consisted of casein¹ 20 parts, brewers' yeast¹ 12 parts, ground rice 67 parts, and salt mixture 1 part. To 100 gm. of this mixture, 1 cc. of cod liver oil (U. S. P.) was added immediately before feeding. The mixture was fed *ad libitum*. The salt mixture had the following composition: calcium carbonate 48 parts, sodium chloride 48 parts, copper sulfate 0.2 part, ferrous sulfate 1.4 parts, manganous sulfate 1.0 part, and potassium chloride 1.4 parts.

Several varieties of chicks (white Leghorns, white Rock, etc.) were used with equally satisfactory results. The newly hatched chicks were placed in an electrically heated brooder with a wire mesh floor. The drinking water contained 0.1 per cent benzoic acid to inhibit growth of bacteria.

Determination of Prothrombin Time

Blood was collected from the wing vein with a 1 cc. syringe and a No. 27 needle. The syringe into which 0.03 cc. of 0.1 M sodium oxalate was put was chilled before use. Approximately 0.3 cc. of blood was drawn and immediately transferred to a small test-tube immersed in an ice bath, and centrifuged. Sufficient plasma was usually obtained for two determinations. The prothrombin time was determined exactly as outlined by Quick (7). The thromboplastin was prepared from chicken brain dehydrated with acetone.

The stock solutions of menadione and vitamin K₁ were prepared as follows: For menadione, 0.2 gm. of 2-methyl-1,4-naphthoquinone was dissolved in 100 cc. of alcohol (1 cc. = 2 mg.). For vitamin K₁, 0.5 gm. of vitamin K (Merck) was dissolved in 100 cc. of alcohol (1 cc. = 5 mg.). Both solutions were protected from light and kept in a refrigerator. To prepare the working solution, 1 cc. of the stock solution was mixed with an appropriate amount of distilled water, so that the concentration was

¹ Vitamin-tested casein and brewers' yeast obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

such that the desired amount to be administered to a chick was contained in 0.1 to 0.5 cc. On diluting the alcoholic solution of vitamin K₁, a milky suspension was obtained, which was frequently shaken to maintain a uniform concentration. The dilute aqueous solution was given orally to the chick by means of a serological pipette.

Comparison of Activity of Menadione and Natural Vitamin K₁

To compare the relative potency of 2-methyl-1,4-naphthoquinone and vitamin K₁, chicks 1 day old were put on the vitamin K-free diet and given a fixed amount of the vitamin K preparation daily. It was found convenient to work with five to six chicks in a group. Blood was taken on the 10th or 11th day and analyzed for prothrombin concentration. The results are recorded in Table I.

It is clear even from a casual inspection of results that the method is sensitive to changes of less than 0.5 γ of menadione. Between 1.5 and 2 γ per day are required to maintain a normal range of prothrombin in a chick during the first 10 days of life. This finding is completely in agreement with the report of Stamler *et al.* (6). It is to be noted that there always appears to be good agreement between the one- and two-stage method in uncomplicated vitamin K deficiency.

Little vitamin K is stored at the 2 γ level, for on withdrawing the vitamin for 2 days a marked drop in prothrombin occurs. Interesting, but unexplainable, is the observation that the lower the prothrombin the less pronounced is the relative fall after withdrawal of the vitamin. When more than 2 γ per day was given, storage occurred and the prothrombin level remained normal for several days after withdrawal of vitamin K from the diet.

The minimal amount of natural vitamin K required to keep the prothrombin level normal is slightly more than 2.5 γ . Dam, Glavind, and Karrer (8) in their early studies found that natural vitamin K₁ was only one-half as potent as 2-methyl-1,4-naphthoquinone. Since the molecular weight of natural vitamin K₁ is 2.5 times that of menadione, it appears that the body utilizes only the methylnaphthoquinone part of the molecule, and therefore it is logical that a 2.5:1 ratio in activity should be observed. Actually, natural vitamin K₁ on a molecular basis is somewhat more effective than menadione, probably because it is less easily excreted and therefore is more completely utilized. Either natural vitamin K₁ or menadione could be employed as a standard of reference, as suggested by Thayer and his associates (9).

Speed of Utilization of Vitamin K

Dam and his associates noted that vitamin K₁ injected intravenously did not immediately restore the prothrombin level, but required at least 5

PROTHROMBIN LEVEL OF BLOOD. IV

hours (10). Similar results were obtained in the present study with 2-methyl-1,4-naphthoquinone. Interestingly, a minimal effective dose is required which is a little less than 5 γ . The speed of prothrombin produc-

TABLE I
Relative Potency of 2-Methyl-1,4-naphthoquinone and Natural Vitamin K₁

Chick No.	Prothrombin time				
	2-Methyl-1,4-naphthoquinone daily				
	0	0.5 γ	1.0 γ	1.5 γ	2.0 γ
	sec.	sec.	sec.	sec.	sec.
1	59	18	14	13	11
2	100	16	14	11	11
3	51	14	18	12½	11
4	55	27	14	12	11
5	63	25	15	13½	11
Average at end of 11 days*	66 (4)	20 (18)	15 (30)	12 (60)	11 (100)
1		21	20	20	14
2		26	20	19	14
3		28	30	18	14
4		46	24	21	14
Average at end of 13 days†		31 (8)	23½ (11)	19½ (15)	14 (40)
	Vitamin K ₁ daily				
	0	1.25 γ	2.5 γ	3.75 γ	5.0 γ
1	33	13	12	11	11½
2	52	16	13	11	11
3	54	18	11	11	11½
4	47	13½	13½	11	10½
5	55	14	11½	11½	11
Average at end of 11 days	48 (5)	15 (30)	12 (60)	11 (100)	11 (100)

* The figures in parentheses are the calculated concentrations of prothrombin in per cent of normal.

† No vitamin K was administered after the 11th day.

tion is not increased by administering larger doses. Thus, the recovery of prothrombin activity after the injection of 10 γ of menadione was the same as when 5 γ were given. It is to be observed (Fig. 1) that the action of

vitamin K is slight during the 1st hour, then becomes accelerated, and is complete in 4 hours. If the reaction is stoichiometric, it can be concluded

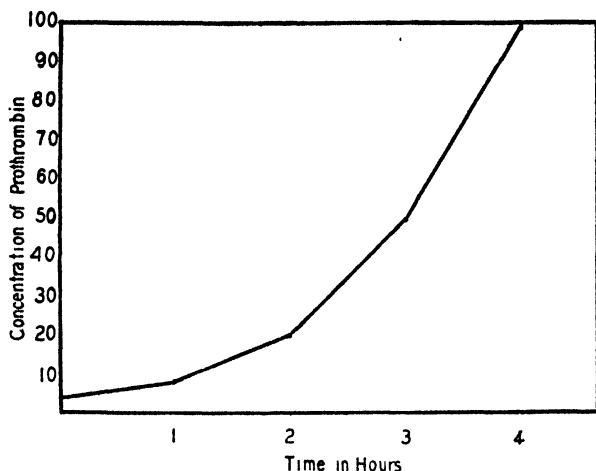


FIG. 1. The prothrombin response to the intravenous injection of 2-methyl-1,4-naphthoquinone in a chick suffering from vitamin K deficiency. The dose injected was 5 γ . The response was of the same order when 10 γ were given.

TABLE II

Counteracting Effect of 2-Methyl-1,4-naphthoquinone and Vitamin K₁ on Hypoprothrombinemia Induced by Dicumarol

Prothrombin time						
Dicumarol, mg. per kg. body weight	20	20	20	20	20	
2-Methyl-1,4-naphthoquinone, micrograms.	5	1	5	2.5		
Natural vitamin K ₁ , micrograms.						
	sec.	sec.	sec.	sec.	sec.	sec.
Chick 1.....	13	32	25	45	45	35
" 2.....	16	28	28	25	74	68
" 3.....	13	40	25	63	71	51
" 4.....	12½	36	25	44	85	87
" 5.....	11½	34	27	39	95	55
Average*.....	13 (50)	34 (7)	26 (10)	43 (5)	74 (3)	59 (4)

* The figures in parentheses represent the concentration of prothrombin in per cent of normal.

that the prothrombin in 1 cc. of blood requires approximately 1 γ of the methylnaphthoquinone radical,

Influence of Vitamin K on Action of Dicumarol

Considerable evidence has been obtained from animal and clinical studies that both natural and synthetic vitamin K counteract the action of dicumarol (11). By using chicks on a controlled diet, the action of dicumarol can be satisfactorily studied in relation to vitamin K.

From the results recorded in Table II it can be seen that chicks on a vitamin K-free diet are more susceptible to the action of dicumarol than

Influence of Hypervitaminosis A on Prothrombin Level of Chicks Maintained on Controlled Vitamin K Intake

Chick No.	Prothrombin time					Remarks
	Vitamin A units daily					
	50,000	30,000	20,000	10,000		
	sec.	sec.	sec.	sec.	sec.	
1	10½	11	14	10½	12½	Each chick received 1.5 γ 2-methyl - 1,4-naphthoquinone daily
2	9½	10	12½	11	9½	
3	10½	11½	11½	12	10½	
4	11	9½	12	12½	10½	
5	11	10½	11	11	13	
Average*	10½ (100)	10½ (100)	12 (60)	11½ (70)	11 (80)	
1	14	19	13	12½	12½	Each chick received 2.5 γ vitamin K ₁ daily
2	13	12	12	18	17	
3	15	15½	12½	18	20	
4	19	12	20	19	15	
5	17	13½	13½	13	14	
Average*	15½ (28)	14½ (35)	14 (40)	16 (25)	15½ (28)	

* The figures in parentheses represent the concentration of prothrombin in per cent of normal.

those receiving either the natural or synthetic vitamin. Surprisingly, however, even with the large dose of dicumarol, 20 mg. per kilo of body weight, the prothrombin decrease is not greater than in dogs on a normal diet and receiving a smaller dose of dicumarol. The resistance of hens to dicumarol has been noted previously (12). The larger the dose of 2-methyl-1,4-naphthoquinone, the less marked the hypoprothrombinemia from dicumarol. There appears to be no marked difference between the effectiveness of menadione and natural vitamin K₁ in counteracting the action of dicumarol in chicks.

Effectiveness of Vitamin K When an Excessive Amount of Vitamin A Is Administered

Light, Alscher, and Frey (12) observed that when rats were fed large doses of Vitamin A (35,000 to 40,000 units per day) a marked fall in prothrombin occurred which could be prevented by administering natural vitamin K₁. These results have been confirmed by Walker, Eyllenburg, and Moore (13), who found synthetic vitamin K₁ likewise counteracted the hypoprothrombinemic effect of vitamin A. Maddock, Wolbach, and Jensen also have reported on hypoprothrombinemia following hyper-vitaminosis A (14).

The action of vitamin A in producing hypoprothrombinemia is not understood. Whether it inhibits the synthesis of vitamin K by the intestinal flora or interferes in the absorption or utilization of vitamin K cannot be determined by using rats which normally depend on bacterial action for part of their vitamin K requirement. By employing chicks on a vitamin K-free diet and giving them a daily amount of vitamin K which is slightly less than the dose needed to maintain the prothrombin level normal, the effect of excessive doses of vitamin A can readily be determined. It will be observed (Table III) that even large doses of vitamin A had no demonstrable effect on the prothrombin time. In the chick, therefore, vitamin A does not interfere either with the absorption or utilization of vitamin K. Since the chick is dependent entirely on the fixed amount of the subnormal dose of vitamin K administered, any action of vitamin A would readily be reflected in the prothrombin level. Thus it seems probable that the hypoprothrombinemic action of vitamin A in the rat is likely to be due to its interference in the synthesis of vitamin K by the intestinal flora.

SUMMARY

1. A simple vitamin K-free diet which produces a marked hypoprothrombinemia in newly hatched chicks is described.
2. A method for vitamin K assay is outlined.
3. It was found that 1.5 to 2 γ of 2-methyl-1,4-naphthoquinone or a little more than 2.5 γ of natural vitamin K₁ per day are required to maintain the prothrombin level of a chick during the first 10 days of life.
4. On injecting menadione intravenously into markedly deficient chicks, the prothrombin activity returns slowly at first, then progressively more rapidly, and becomes normal after 4 hours. The size of dose beyond the minimal effective dose does not influence the speed of recovery of prothrombin activity.
5. The susceptibility of the chick to the action of dicumarol is increased by removal of vitamin K from the diet. Addition of vitamin K counter-

acts the hypoprothrombinemic effect of dicumarol. No marked difference in effectiveness between natural vitamin K₁ and menadione could be demonstrated.

6. Excessive intake of vitamin A, which causes a hypoprothrombinemia in rats, has no effect on the prothrombin level of chicks receiving synthetic or natural vitamin K₁ in doses slightly less than that required for maintaining the prothrombin concentration completely normal.

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STUDIES ON THE POSSIBLE MULTIPLE NATURE OF DEHYDROPEPTIDASE I

BY JESSE P. GREENSTEIN, VINCENT E. PRICE, AND
FLORENCE M. LEUTHARDT

(From the National Cancer Institute, National Institute of Health, Bethesda,
Maryland)

(Received for publication, June 8, 1948)

Dehydropeptidase I is the designation which has been given the enzyme in animal and plant tissues which hydrolyzes glycyldehydroalanine (1-4). A purified preparation of an enzyme with a high activity toward this substrate has been obtained by Shack from beef kidney.¹ Inasmuch as glycyldehydrophenylalanine and DL-alanyldehydroalanine are also hydrolyzed by tissue extracts (3) and by the purified enzyme,¹ the problem arises as to whether all three substrates are hydrolyzed by the same enzyme whose basic specificity requirements involve an α -nitrogen atom adjacent to the dehydropeptide bond.

The present communication describes certain experiments which bear on this problem, and includes the synthesis of certain new dehydropeptides.

EXPERIMENTAL

DL-Alanyldehydrophenylalanine was prepared by the general procedure developed by Bergmann and coworkers (5), which involves the hydrolysis of the azlactone formed from α -chloroacylated phenylserine. The N-methyl derivatives of alanyldehydrophenylalanine and of glycyldehydrophenylalanine were prepared by treating the respective chloro derivatives with cold methylamine. A low temperature was employed to avoid the possibility of addition of methylamine to the double bond of the dehydropeptide (4).

dl-Chloropropionylphenylserine—*dl*-Chloropropionyl chloride was prepared by treating 100 gm. of *dl*-chloropropionic acid with 200 gm. of thionyl chloride. The mixture was boiled for 3 hours under a reflux condenser with careful exclusion of moisture. At the end of this period the mixture was fractionated by distillation *in vacuo*, and that fraction taken which boiled at 40-45° under 25 mm. pressure.

100 gm. of phenylserine were dissolved in 250 cc. of chilled 2 N NaOH, and with further chilling 76 gm. of *dl*-chloropropionyl chloride and 400 cc. of 2 N NaOH were added alternately and with shaking. At the end of the reaction, concentrated HCl was slowly added to the mixture to pH 2.0. A

¹ Shack, J., personal communication.

dense white oil appeared which rapidly hardened to a granular mass. The product was washed at the pump several times with cold water, and crystallized from 60 per cent alcohol in the form of stout prisms. The yield was 68 gm., and the material melted sharply at 161°.

$C_{12}H_{14}O_4NCl$. Calculated, N 5.2, Cl 13.1; found, N 5.3, Cl 13.1

dl-Chloropropionyldehydrophenylalanine—50 gm. of recrystallized and thoroughly dried chloropropionylphenylserine were suspended in 500 cc. of acetic anhydride and the mixture kept in a boiling water bath for 3 hours. After about $\frac{1}{2}$ hour complete solution had occurred, and with further heating the color changed from yellow to a brownish red. The solution was cooled, and with careful exclusion of moisture evaporated *in vacuo* to a thick red syrup. No crystals of the azlactone appeared after several hours of chilling, and therefore the entire syrupy mass was subjected to a hydrolytic procedure without purification. The syrup was taken up in 250 cc. of acetone, treated with 125 cc. of water, and the turbid mixture boiled under the reflux for 2 hours. After 1 hour of boiling, a clear, red solution resulted. The solution was cooled and extracted twice with ligroin. Much of the color went into the ligroin layer, and the product crystallized quickly from the aqueous layer. The product was filtered off and washed several times with water. It was recrystallized from acetone and appeared in tufts of long needles. The crystalline material was filtered at the pump and washed with dry ether, which served to remove the last trace of color. The yield was 20 gm., and the melting point was 207°.

$C_{12}H_{12}O_3NCl$. Calculated, N 5.6, Cl 14.0; found, N 5.7, Cl 13.7

DL-Alanyldehydrophenylalanine—10 gm. of *DL*-chloropropionyldehydrophenylalanine were dissolved in 100 cc. of ammonia water which had been saturated at 5°. After standing for 3 days at 25°, the solution was evaporated *in vacuo* to dryness, the product dissolved in the minimum amount of hot water, and the hot aqueous solution filtered and treated with an excess of hot absolute alcohol. On chilling for several hours, the dehydropeptide crystallized in the form of long needles. The yield was 4 gm. The compound began to darken at 200°, but did not melt up to 230°.

$C_{12}H_{14}O_3N_2$. Calculated. C 61.5, H 5.9, N 11.9
Found. " 60.9, " 5.6, " 11.4

N-Methyl-DL-alanyldehydrophenylalanine—10 gm. of *DL*-chloropropionyldehydrophenylalanine were dissolved in 100 cc. of chilled 25 per cent methylamine solution in water, and the mixture kept at 5° for 3 days. The mixture was evaporated *in vacuo* to dryness, the product dissolved in the minimum amount of hot water, and the hot aqueous solution filtered and

treated with an excess of hot absolute alcohol. The product appeared very slowly on chilling and separated after several days in the form of long needles. After the mother liquor was worked up and the fractions combined, the yield was 3 gm. The melting point was 200° with decomposition.

$C_{13}H_{16}O_2N_2$. Calculated. C 62.9, H 6.4, N 11.3
Found. " 62.5, " 6.0, " 11.4

N-Methylglycyldehydrophenylalanine—This compound was obtained by the same procedure as methylalanyldehydrophenylalanine, except that chloroacetyldehydrophenylalanine (6) was employed. From 10 gm. of starting material, 2.5 gm. of recrystallized product in the form of needles were obtained. The melting point was 185° with decomposition.

$C_{12}H_{14}O_2N_2$. Calculated. C 61.5, H 5.9, N 11.9
Found. " 61.5, " 6.0, " 11.9

Chloroacetyl-DL-alanyldehydroalanine—5 gm. of DL-alanyldehydroalanine (3) were dissolved in 20 cc. of chilled 2 N NaOH and with further chilling treated alternately in small portions with 5 gm. of chloroacetyl chloride and 30 cc. of 2 N NaOH. At the end of the reaction, the mixture was acidified with 5 N HCl to a Congo blue reaction and evaporated *in vacuo* to approximately half the volume. The product separated on chilling. It was recrystallized from hot water; m.p. 132°. There was no evidence of crystal water.

$C_8H_{11}O_4N_2Cl$. Calculated. C 40.9, H 4.7, N 11.9, Cl 15.1
Found. " 40.3, " 4.6, " 11.8, " 15.2

Chloroacetylglycyldehydroalanine and Glycylglycyldehydroalanine (Correction)—The synthesis and enzymatic susceptibility of these compounds have been reported (4). Because of certain inconsistencies which developed later as a result of the use of new substrates, it was decided to reexamine our earlier material. We noted that the compound referred to as chloroacetylglycyldehydroalanine was contaminated with glycyldehydroalanine hydrochloride, which renders invalid the enzymatic data obtained, as well as the subsequent amination procedure. We are unable to explain the manner of this contamination, which is unique in our experience. Reexamination of all of our other chloroacylated dehydropeptides failed to reveal any admixture with the hydrochloride of the starting material, or, for that matter, with any significant amount of impurity. Careful repetition of the synthesis described (4) yielded a product which, after recrystallization from water as long prisms, showed no presence of inorganic chloride. M.p. 163° with decomposition.

$C_7H_9O_4N_2Cl$. Calculated. N 12.7, Cl 16.1
Found. " 12.7, " 15.9

Amination in the manner described (4) led to the preparation of glycylglycyldehydroalanine which appeared from water-alcohol mixtures as flat prisms. M.p. 214° with decomposition.

$C_7H_{11}O_4N_3$. Calculated. C 41.8, H 5.5, N 20.9
Found. " 41.8, " 5.6, " 20.7

Other Substrates—Chloroacetylglycyldehydrophenylalanine and glycylglycyldehydrophenylalanine were prepared by the method of Bergmann and Schleich (6). The melting point of the former compound was 210°; that of the latter was 216° with decomposition. The preparation of the other dehydropeptides has been described (1–4). All substrates were stable in water and in the presence of boiled tissue preparations.

Ultraviolet Absorption Spectra of Dehydropeptides—The absorption curves of alanyldehydrophenylalanine, methylalanyldehydrophenylalanine, and methylglycyldehydrophenylalanine are practically congruent with each other and with those of the dehydrophenylalanyl peptides reported earlier (3). For all compounds of this class, there is a characteristic absorption with a maximum at 2750 Å. The molar extinction coefficient at this wavelength is 14,900 for the three compounds dissolved in water. For chloropropionyldehydrophenylalanine and chloroacetylglycyldehydrophenylalanine brought into aqueous solution at pH 7.2 by addition of dilute NaOH, the molar extinction coefficient is 15,220, while for glycylglycyldehydrophenylalanine it is 14,400.

The absorption curves for chloroacetylalanyldehydroalanine, chloroacetylglycyldehydroalanine, and glycylglycyldehydroalanine are practically congruent with those for chloroacetyldehydroalanine and glycyldehydroalanine (3), with a maximum at 2400 Å and a molar extinction coefficient of 6100.

Enzymatic Hydrolysis of Substrates—The hydrolysis of the dehydropeptides (1–4) was measured as heretofore by the rate of appearance of ammonia N at 37° above the controls. The digests consisted of 1 cc. of freshly prepared aqueous rat tissue extract plus 2 cc. of 0.15 M borate buffer at pH 8.1, plus 1 cc. of either water or 0.025 M substrate solution (0.05 M in the case of racemic substrates). The activity is expressed as micromoles $\times 10$ substrate hydrolyzed per hour per mg. of total N in the extract. Since 1 atom of ammonia N is derived from the hydrolysis of one dehydropeptide bond, the data refer directly to the splitting of the substrate. The ammonia measured in digests of the DL-alanyl dehydropeptides is not derived from oxidase activity, since identical results were obtained under anaerobic and aerobic conditions. No digestion lasted longer than 2 hours, and most were 1 hour or less in duration. All solutions of chloroacylated substrates were brought to pH 7.0 with dilute NaOH before use.

The tissues were freshly removed from rats sacrificed by decapitation. Several hepatoma samples, donated by Dr. J. White, were used. These were primary tumors, obtained from Osborne-Mendel rats as a result of feeding *p*-dimethylaminoazobenzene. Necrotic areas of the tumor were carefully dissected and discarded. Values obtained with different samples

TABLE I
Enzymatic Hydrolysis of Various Dehydropeptides

Substrate	Micromoles $\times$ 10 substrate hydrolyzed per hr. per mg. N with					
	Kidney*	Pan- creas	Spleen	Brain	Liver	Hepa- toma
Glycyldehydroalanine	1620	530	331	72	60	380
N-Methylglycyldehydroalanine†	800	280	166	30	25	10
Chloroacetylglycyldehydroalanine	0	0	0	0	0	0
Chloroacetyl-N-methylglycyldehydro- alanine	0	0	0	0	0	0
Glycylglycyldehydroalanine	1040	400	210	30	22	156
DL-Alanyldehydroalanine	1350	722	410	220	52	1250
Chloroacetyl-DL-alanyldehydroalanine†	85	63	54	30	26	21
Glycyldehydrophenylalanine	520	132	23	0	12	0
N-Methylglycyldehydrophenylalanine†	180	32	12	0	6	0
Chloroacetylglycyldehydrophenylalanine	0	0	0	0	0	0
Glycylglycyldehydrophenylalanine	412	88	30	0	12	0
DL-Alanyldehydrophenylalanine†	220	32	14	0	8	0
N-Methyl-DL-alanyldehydrophenylalan- ine†	140	12	6	0	5	0

* DL-Chloropropionyldehydrophenylalanine is not hydrolyzed.

† These compounds are hydrolyzed by the purified dehydropeptidase preparation from beef kidney, with rate values of 2600 to 4800 micromoles of substrate hydrolyzed per hour per mg. of nitrogen.

‡ Unlike alanyldehydroalanine (3), this substrate is not hydrolyzed by human serum, or by the purified dehydropeptidase I preparation from beef kidney (see footnote 1 in the text).

of the tumor agreed within 20 per cent. A few experiments were made with a purified dehydropeptidase I preparation donated by Dr. Shack.

Data on the various substrates are given in Table I, and, to facilitate comparison, earlier data on glycyldehydroalanine, glycyldehydrophenylalanine, and alanyldehydroalanine in normal tissues (3, 4) are included.

Certain facts emerge from the data obtained so far. (a) The peptides of dehydroalanine are generally more susceptible substrates than the peptides of dehydrophenylalanine. (b) Chloroacetylation of glycyldehydroalanine or glycyldehydrophenylalanine removes the susceptibility of these compounds to dehydropeptidase action but not that of DL-alanyldehydroala-

nine.³ (c) Amination of the chloroacetyl-glycine dehydropeptides restores the enzymatic susceptibility, as in glycylglycyldehydroalanine and glycylglycyldehydrophenylalanine.³ (d) The N-methylated dehydropeptides are generally hydrolyzed at a slower rate than the unsubstituted parent compounds. (e) When rat liver becomes cancerous, *i.e.* is transformed into a hepatoma, the rate of hydrolysis of glycyldehydroalanine increases some 6-fold, that of alanyldehydroalanine increases some 20-fold, while that of N-

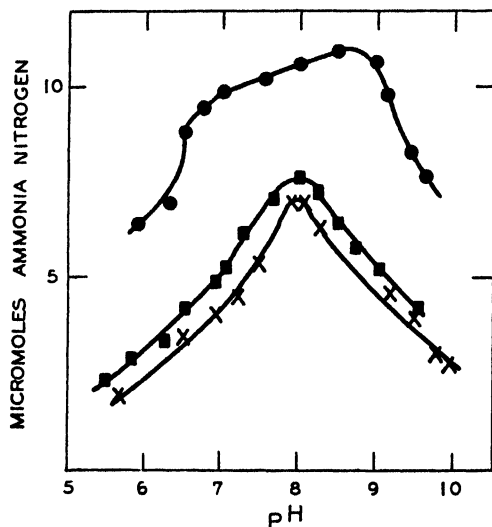


FIG. 1. pH-activity curves for chloroacetyldehydroalanine (●) and glycyldehydroalanine (■) in rat liver extracts, and glycyldehydroalanine (X) in rat hepatoma extracts. Extract concentrations were suitably prepared. Buffers below pH 8.0 were veronal-acetate; above pH 8.0, glycine-NaOH.

methylglycyldehydroalanine decreases and that of all of the peptides of dehydrophenylalanine practically disappears (a property shared by brain; Table I). The high level of activity toward certain aliphatic dehydro-

³ The explanation for the enzymatic resistance of chloroacetyl-glycine dehydropeptides and the susceptibility of glycylglycine dehydropeptides and of chloroacetyl-DL-alanyldehydroalanine may be connected, on the one hand, with the absence in tissue extracts of an enzyme capable of hydrolyzing the peptide bond between chloroacetic acid and glycine, and, on the other hand, with the presence in such tissue extracts of active enzymes capable of rapidly hydrolyzing glycylglycine and chloroacetyl-DL-alanine. The hydrolysis of glycylglycyldehydroalanine and of glycylglycyldehydrophenylalanine at the dehydropeptide bond is accompanied by hydrolysis of the saturated peptide bond (*cf.* also (6)). The relation of the peptidases and the dehydropeptidases will be the subject of a future communication by the authors (*cf.* (9)).

peptides is noted not only in the rat hepatoma but in all tumors studied (cf. (2, 7)).³

Susceptibility of Dehydropeptides As Function of pH—The pH at which maximum hydrolysis of glycyldehydroalanine¹ and glycyldehydrophenylalanine (8) occurs with purified kidney preparations is 8.0, whereas that of

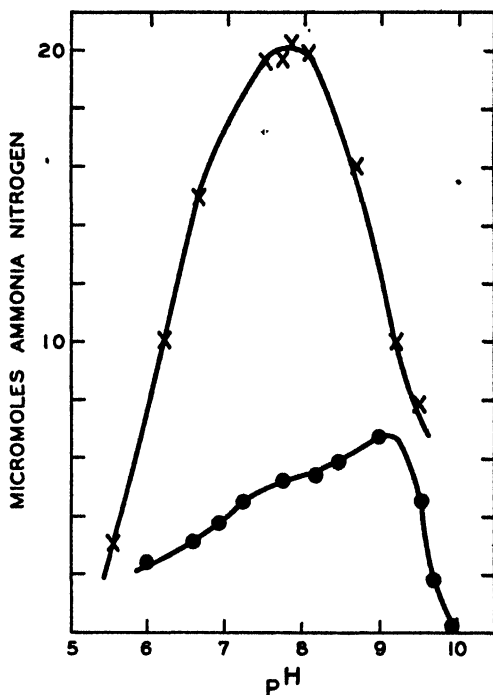


FIG. 2. pH-activity curves for DL-alanyldehydroalanine (X) and for chloroacetyl-DL-alanyldehydroalanine (●) in rat liver digests. 0.050 M concentration of substrates was used.

chloroacetyldehydroalanine is at 9.0.¹ Substantially similar pH-activity curves, respectively, have been obtained for glycyldehydroalanine and

³ We have investigated a wide variety of primary and transplanted tumors in experimental animals, including mammary adenocarcinomas, fibrosarcomas, melanomas, and epitheliomas, and invariably noted the ability of these tissues to hydrolyze alanyldehydroalanine at a rate approximating that of the normal kidney but no apparent ability to attack peptides of dehydrophenylalanine. Although our observations have been limited so far to tumors in laboratory animals, we are inclined to believe that the extraordinary ability of such tumors to hydrolyze alanyldehydroalanine at a rate close to that of the most active normal tissue is as characteristic a property of cancer tissues as are their glycolytic behavior and low oxidase activity. These findings will be discussed in detail elsewhere.

chloroacetyldehydroalanine when incubated with rat liver extracts (Fig. 1). The optimum susceptibility of glycyldehydroalanine in hepatoma digests is the same as in normal liver digests. The pH-activity curve of DL-alanyldehydroalanine resembles that of glycyldehydroalanine and glycyldehydrophenylalanine (relatively sharp optimum at pH 8.0), whereas the curve for chloroacetyl-DL-alanyldehydroalanine resembles that of chloroacetyldehydroalanine (maximum at pH 9.0) (Fig. 2).

The pH-activity curves in Fig. 3, performed with rat kidney extracts, reveal an optimum at pH 8.0 for glycylglycyldehydroalanine, DL-alanyldehydrophenylalanine, N-methyl-DL-alanyldehydrophenylalanine, and N

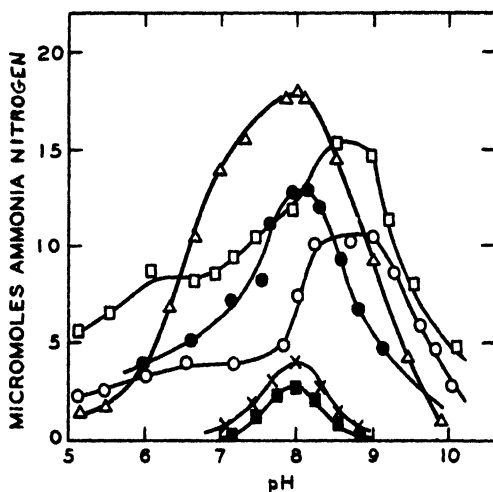


FIG. 3. pH-activity curves in rat kidney digest of methylglycyldehydrophenylalanine (○), DL-alanyldehydrophenylalanine (X), methyl-DL-alanyldehydrophenylalanine (■), methylglycyldehydroalanine (●), glycylglycyldehydrophenylalanine (□), and glycylglycyldehydroalanine (Δ).

methylglycyldehydroalanine, and an optimum at pH 8.8 for glycylglycyldehydrophenylalanine and N-methylglycyldehydrophenylalanine.

The position of the various pH-activity curves in Figs. 1 to 3 does not reflect the relative susceptibility of the substrates, for the extracts in each case were suitably diluted so that initial reaction rates were obtained over the pH range studied.

DISCUSSION

The pH-activity curves describing the susceptibility of the dehydropeptides appear to fall into three more or less characteristic groups: (a) those with a relatively sharp optimum at pH 8.0, including glycyldehydroalanine,

methylglycyldehydroalanine, glycyldehydrophenylalanine, alanyldehydroalanine, glycylglycyldehydroalanine, alanyldehydrophenylalanine, and methylalanyldehydrophenylalanine; (b) those with a relatively broad zone between pH 6 and 8.8, with an optimum at pH 8.8, including glycylglycyldehydrophenylalanine and methylglycyldehydrophenylalanine; and (c) those with a relatively broad zone between pH 7 and 9, with an optimum at pH 9.0, including chloroacetyldehydroalanine and chloroacetylalanyldehydroalanine (Figs. 1 to 3).

It does not appear entirely probable that glycylglycyldehydrophenylalanine is hydrolyzed by the same enzyme system as is glycylglycyldehydroalanine, nor methylglycyldehydrophenylalanine by the same system which hydrolyzes methylglycyldehydroalanine.

The first indication that the enzymes concerned with the hydrolysis of glycyldehydroalanine and glycyldehydrophenylalanine may be different is derived from the observation that Shack's preparation of dehydropeptidase II possesses a small but definite capacity to hydrolyze the former substrate but no capacity to hydrolyze the latter substrate.¹

Perhaps the most striking distinction in the enzymatic actions studied is illustrated by the differences in hydrolysis of the substrates in liver and in hepatoma (Table I). The marked divergence in susceptibility of the substrates as a result of the malignant transformation of the liver appears to set off (a) the enzyme systems which act on the aliphatic from those which act on the aromatic dehydropeptides, and (b) the system or systems which act upon N-methylated glycyldehydroalanine from those which act upon the unsubstituted parent compound. In suggesting that these systems are distributed differently in liver and hepatoma, the tacit assumption is made that the enzyme which attacks glycyldehydroalanine in liver, for example, is qualitatively the same as that which attacks this substrate in the hepatoma (*cf.* Fig. 1).

From the present evidence, therefore, it would appear that the number of dehydropeptidases may be more than the two designated as I and II respectively (1-3). For convenience and consistency, the designations of dehydropeptidase I and II have been retained, I referring to a possible series of enzymes which attack dehydropeptides containing a nitrogen atom in the α position to the dehydropeptide bond, II referring to the enzyme or enzymes which attack dehydropeptides lacking the α -nitrogen atom (4). On this basis, dehydropeptidase I may be subdivided into activities concerned (a) with glycyldehydroalanine, (b) with alanyldehydroalanine, (c) with dipeptides of dehydrophenylalanine, and (d) with N-methylamino dehydropeptides. These four categories of substrates are all hydrolyzed by the purified enzyme preparation of Shack.¹ Since this preparation is particulate in nature, further fractionation might conceivably be difficult.

The difference in the pH-activity curves for methylglycyldehydroalanine and for methylglycyldehydrophenylalanine (Fig. 3) suggests that perhaps (d) may be further subdivided. For the time being, the distribution in various tissue preparations of the capacity to hydrolyze these four main categories of dehydropeptides remains the chief clue to the possible multiple nature of dehydropeptidase I.

SUMMARY

DL-Alanyldehydrophenylalanine, N-methyl-DL-alanyldehydrophenylalanine, N-methylglycyldehydrophenylalanine, chloroacetyl-DL-alanyldehydroalanine, chloroacetylglycyldehydroalanine, and glycylglycyldehydroalanine were synthesized, and together with other dehydropeptides previously described investigated in digests with various rat tissues.

Although chloroacetylalanyldehydroalanine is hydrolyzed by all tissues studied, chloroacetylglycyldehydroalanine and chloroacetylglycyldehydrophenylalanine are resistant. Amination of the last two compounds to the corresponding glycyl derivatives restores susceptibility to enzymatic action. N-Methylated dehydropeptides are hydrolyzed at a slower rate than the parent, unsubstituted compounds.

The rates of hydrolysis of glycyldehydroalanine and of alanyldehydroalanine by the rat hepatoma are, respectively, about 6-fold and 20-fold that of normal liver. Neither hepatoma nor brain appears to have any capacity to hydrolyze peptides of dehydrophenylalanine. The capacity of liver to hydrolyze N-methylglycyldehydroalanine diminishes when this tissue becomes neoplastic.

The pH-activity functions of the various dehydropeptides in digests of rat liver and kidney were determined and appeared to involve roughly three categories of substrates with optimum susceptibilities at pH 8.0, 8.8, and 9.0.

The possible multiple nature of dehydropeptidase I is discussed.

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DERIVATIVES OF α,α -DI(GLYCYLAMINO)PROPIONIC ACID

By JESSE P. GREENSTEIN AND VINCENT E. PRICE

(From the National Cancer Institute, National Institute of Health, Bethesda, Maryland)

(Received for publication, June 8, 1948)

α,α -Di(glycylamino)propionic acid hydrochloride, $(\text{NH}_2\text{CH}_2\text{CONH})_2\text{C}(\text{CH}_3)\text{COOH}\cdot\text{HCl}$, is enzymatically hydrolyzed in aqueous extracts of rat kidney to yield, as a maximum, 1 mole of ammonia and 1 mole of pyruvic acid per mole of substrate (1). α -(*dl*-Chloropropionylamino)- α -(*DL*-alanyl-amino)propionic acid and α,α -di(*DL*-alanylamino)propionic acid hydrochloride are also hydrolyzed by rat kidney, the former substrate yielding a molar ratio of ammonia to pyruvic acid close to unity, the latter a ratio close to 1.6 (2). On the other hand, α,α -di(acetamino)propionic acid, α,α -di(chloroacetyl-amino)propionic acid, and α,α -di(*dl*-chloropropionylamino)propionic acid are completely resistant (1, 2).

Investigations on this novel class of substances have been carried further by observing whether there is any relation between the integrity of the α -amino groups of di(glycylamino)propionic acid and susceptibility to enzymatic attack by a variety of rat tissues. Several new derivatives of di(glycylamino)propionic acid have been synthesized.

EXPERIMENTAL

α,α -Di(*N*-methylglycylamino)propionic Acid Hydrochloride—10 gm. of α,α -di(chloroacetyl-amino)propionic acid (1) were dissolved in 100 cc. of a 25 per cent aqueous solution of methylamine, and the solution kept at 40° for 2 days. On evaporation *in vacuo* a yellow oil was obtained, which, on being shaken with absolute alcohol for several hours, yielded a mass of fine white crystals. The material was filtered at the pump, dissolved in hot water, and recrystallized by the cautious addition of hot alcohol. The product crystallized in tufts of long needles. M.p. 215° with decomposition; yield 3.5 gm.

$\text{C}_8\text{H}_{18}\text{O}_4\text{N}_4\cdot\text{HCl}$. Calculated. C 38.2, H 6.8, N 19.8, Cl (ionic) 12.5
Found. " 38.0, " 6.4, " 19.9, " " 12.1

α,α -Di(*N*-dimethylglycylamino)propionic Acid Hydrochloride—The preparation of this product followed essentially the same procedure as that described for the monomethyl derivative above, except that a 25 per cent aqueous solution of dimethylamine was employed. The product crystal-

lized in the form of long needles. M.p. 219° with decomposition; yield 5.2 gm.

$C_{11}H_{22}O_4N_4 \cdot HCl$. Calculated. C 42.5, H 7.4, N 18.0, Cl (ionic) 11.4
Found. " 41.8, H 7.3, " 17.7, " " 11.2

α, α -Di(chloroacetylglcyclamino)propionic Acid—20 gm. of α, α -di(glycylamino)propionic acid hydrochloride (1) were dissolved in 100 cc. of chilled 2 N NaOH and, with further cooling, treated alternately and with shaking with 30 gm. of chloroacetyl chloride and 200 cc. of 2 N NaOH. At the end of the reaction the mixture was treated with 5 N HCl to pH 2.0, filtered, and evaporated *in vacuo* to dryness. The residue was extracted with hot acetone and filtered. To the cooled filtrate ethyl acetate was added dropwise. The first oily precipitate was discarded, and with further addition of ethyl acetate a yellowish, granular precipitate appeared. The material was filtered off and washed several times with dry ether, which removed the last traces of color. The product was redissolved in acetone and treated again with ethyl acetate. Repetition of this procedure yielded 12 gm. of a white, powdery product which possessed no definite crystal form; m.p. 98°.

$C_{11}H_{18}O_6N_4Cl_2$. Calculated. C 35.6, H 4.3, N 15.1, Cl 19.1
Found. " 35.7, " 4.4, " 14.4, " 18.1

α, α -Di(glycylglycyclamino)propionic Acid Hydrochloride—2 gm. of di(chloroacetylglcyclamino)propionic acid were dissolved in 40 cc. of 28 per cent ammonia and kept in a sealed flask for 36 hours at 40°. At the end of this period the solution was evaporated at 20° to dryness, and the residue washed several times with alcohol to remove ammonium chloride. The compound was then taken up several times in the minimum amount of water and precipitated each time with an excess of absolute alcohol. A white powder was finally obtained which had no definite crystal form. Yield 1.1 gm.; m.p. 164° with decomposition.

$C_{11}H_{20}O_6N_4 \cdot HCl$. Calculated. N 22.7, Cl (ionic) 9.6
Found. " 21.9, " " 9.3

The enzymatic susceptibility of the di(acylamino)propionic acids was tested in the manner described (1, 2). All substrates were stable in aqueous solution and in the presence of boiled tissue preparations. The digests consisted of 1 cc. of freshly prepared aqueous rat tissue extract, 2 cc. of 0.15 M borate buffer at pH 8.1, and 1 cc. of either water or 0.025 M substrate solution. The extracts were prepared by grinding the tissues with clean sand and homogenizing with distilled water, followed by light centrifugation. The rate at which ammonia and pyruvic acid appeared over that of the controls, during the period when such a rate was nearly linear, was taken as a measure of enzymatic activity. 1 mole of ammonia appeared per mole of substrate hydrolyzed (1). The temperature of digestion was

37°. Solutions of di(chloroacetylglycylamino)propionic acid were brought to pH 7.0 with dilute NaOH before addition to the digests. The data are given in Table I.

DISCUSSION

Di(glycylamino)propionic acid, in effect, may be considered as glycyldehydroalanine to which a mole of glycineamide has been added at the double bond (1). For this reason, and because the participation of dehydropeptidase has been suspected in the enzymatic degradation of the di(acylamino)-propionic acids (1, 2), a comparison of the susceptibility of analogously

TABLE I
Enzymatic Hydrolysis of Di(glycylamino)propionic Acid and Derivatives in Rat Tissue Extracts

Substrate	Micromoles $\times$ 10 ammonia N evolved per hr. per mg. total N in extract of					
	Kidney	Pancreas	Spleen	Brain	Liver	Hepatoma
α, α -Di(glycylamino)propionic acid†	50	15	8	3	2	5
α, α -Di(N-methylglycylamino)propionic acid†	24	8	3	1	1	0
α, α -Di(N-dimethylglycylamino)propionic acid	0	0	0	0	0	0
α, α -Di(chloroacetylglycylamino)propionic acid	0	0	0	0	0	0
α, α -Di(glycylglycylamino)propionic acid	38	14	8	2	1	2

* Primary tumors induced in Osborne-Mendel rats by feeding *p*-dimethylaminoazobenzene; donated by Dr. J. White.

† Pyruvic acid determined in the digests as heretofore (1) was practically equimolar with the ammonia evolved.

constituted dehydropeptides of alanine and di(acylamino)propionic acids is of interest (3).

For both dehydropeptides and di(acylamino)propionic acids, the most active normal tissue is kidney, followed in descending order by pancreas, spleen, brain, and liver. The susceptibility of both glycyldehydroalanine and di(glycylamino)propionic acid is higher in extracts of the hepatoma than in those of normal liver. The susceptibility in normal tissue extracts of both N-methylglycyldehydroalanine and di(N-methylglycylamino)propionic acid is roughly half that of their respective unsubstituted parent compounds, while in the hepatoma extract the susceptibility of the N-methylated compounds practically vanishes. No comparison can be made of the N-dimethyl analogues because of the failure to synthesize N-dimethylglycyldehydroalanine (4). Like chloroacetylglycyldehydroalanine,

di(chloroacetylglycylamino)propionic acid is not apparently enzymatically hydrolyzed. When, however, both compounds are aminated, to form the respective glycylglycyl derivatives, the latter are again enzymatically susceptible. These findings may be related to the absence in tissues of an enzyme capable of hydrolyzing the bond between chloroacetic acid and glycine and to the presence of a peptidase which rapidly hydrolyzes the bond in glycylglycine (*cf.* (3)).

The enzymatic hydrolysis of di(glycylamino)propionic acid has been considered to involve either one or perhaps both of the following mechanisms (1): (a) an initial enzymatic hydrolysis at one of the two glycylamino linkages, leading to the formation of glycine and the unstable α -amino- α -glycylaminopropionic acid which spontaneously decomposes to ammonia, glycineamide, and pyruvic acid, or (b) an initial attack at the bond between a glycylamino residue and the tertiary carbon atom, leading to the formation of glycineamide and glycyldehydroalanine, the latter being subsequently hydrolyzed by dehydropeptidase to glycine, ammonia, and pyruvic acid.¹ The end-products of the hydrolysis are presumably the same by either mechanism. The similarity in susceptibility of analogously constituted dehydropeptides and di(glycylamino)propionic acid derivatives in normal tissues and in the hepatoma appears to support mechanism (b), but this similarity, although interesting, may possibly be deceptive. Studies of variously constituted substrates with different tissues are capable of providing some illumination but are not decisive. It appears highly reasonable that the hydrolysis of the di(acylamino)propionic acids occurs in two consecutive steps, and that the initial reaction requires in the susceptible substrate a basic α -nitrogen atom to which at least 1 hydrogen atom is attached. The problem concerned with the course of this hydrolysis will be solved when both steps are separated and individually characterized. Work in this direction is being pursued by parallel studies on saturated peptides with purified tissue fractions.

SUMMARY

α, α -Di(N-methylglycylamino)propionic acid hydrochloride, α, α -di(N-dimethylglycylamino)propionic acid hydrochloride, α, α -di(chloroacetyl-

¹ Using the ninhydrin- CO_2 procedure of following peptide hydrolysis (5), we have noted an appreciable rate of hydrolysis of glycylaminoisobutyric acid in rat kidney digests. In terms of micromoles $\times 10$ of substrate hydrolyzed per hour per mg. of N in the extract, the rate is 620. This compound, $\text{NH}_2\text{CH}_2\text{CONHC}(\text{CH}_3)_2\text{COOH}$, whose structural analogy to di(glycylamino)propionic acid may be noted, is hydrolyzed almost exclusively at the peptide bond. No ammonia is evolved in kidney digests of glycylaminoisobutyric acid (6). In calculating the rate of hydrolysis of glycylaminoisobutyric acid, cognizance must be taken of the fact that aminoisobutyric acid itself contributes about 12 per cent to the total carbon dioxide evolved on complete hydrolysis of the peptide. This amino acid apparently does not react completely with ninhydrin under the conditions employed.

glycylamino)propionic acid, and α, α -di(glycylglycylamino)propionic acid hydrochloride were prepared, and their hydrolysis in various rat tissue extracts followed mainly by the rate of ammonia evolution over the controls. The monomethylglycyl and glycylglycyl derivatives were hydrolyzed by all normal rat tissues studied, whereas the dimethylglycyl and chloroacetylglycyl derivatives were not attacked. In extracts of hepatoma, the susceptibility of di(glycylamino)propionic acid is higher than in liver, whereas that of di(N-methylglycylamino)propionic acid nearly vanishes. Comparison is made between analogously constituted dehydropeptides and di(glycylamino)propionic acid derivatives, and certain similarities are described.

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ENZYMATIC HYDROLYSIS OF ANALOGOUS SATURATED AND UNSATURATED PEPTIDES

By VINCENT E. PRICE AND JESSE P. GREENSTEIN

(From the National Cancer Institute, National Institute of Health, Bethesda, Maryland)

(Received for publication, June 8, 1948)

Two types of peptidases are present in tissues, one of which catalyzes the hydrolysis of the saturated, $\text{RCHCONHCHR}'\text{COOH}$,¹ the other that of the unsaturated, $\text{RCHCONH}(\text{=CHR}')\text{COOH} \rightleftharpoons \text{RCHCON}=\text{C}(\text{CH}_2\text{R}')\text{COOH}$, peptide bonds (cf. (1)). These are designated, respectively, peptidases and dehydropeptidases. We have reported the rates of hydrolysis of variously constituted dehydropeptides in extracts of rat tissues (2-4). The present study consists in a comparison of such rates with those of analogous saturated peptides under nearly identical experimental conditions. Comparison has been made between glycyl-DL-alanine and glycyl-dehydroalanine, glycyl-DL-phenylalanine and glycyldehydrophenylalanine, and chloroacetyl-DL-alanine and chloroacetyldehydroalanine, studied in extracts of rat kidney, liver, and hepatoma. Acetyl-DL-alanine, chloroacetyl-DL-phenylalanine, acetylglycine, and glycylglycine were also studied in kidney digests, and the former two compounds compared, respectively, with acetyldehydroalanine and chloroacetyldehydrophenylalanine.

EXPERIMENTAL

The digests were composed of 1 cc. of fresh, aqueous rat tissue extract,² 1 or 2 cc. of 0.15 M borate buffer at pH 8.0 and 1 cc. of either water or 0.025 M neutralized dehydropeptide or 0.050 M neutralized racemic peptide solution. No metallic or other activator was added. The hydrolysis at 37° of the dehydropeptides was followed by the rate of evolution of ammonia (2) and that of the peptides by measuring the CO_2 evolved after treatment with ninhydrin (5), both rates being corrected for the respective

¹ Substrates containing an α tertiary carbon atom, such as glycylaminoisobutyric acid and favorably constituted di(acylamino)propionic acids, are also hydrolyzed in rat tissues (14). The relation, if any, between the enzymes acting upon such substrates and those catalyzing the hydrolysis of peptides with a hydrogen atom on the α -carbon is not yet clear.

² The extracts were prepared by grinding the freshly removed tissue with clean sand in a glass mortar and suspending in water, followed by light centrifugation to remove the sand and tissue debris. The extracts were used within a half hour of preparation.

controls.³ In agreement with the opinion expressed by Neurath *et al.* (6), we have found the specific and accurate ninhydrin method the most satisfactory of contemporary procedures for measuring the rate of peptide hydrolysis.

The saturated peptides employed were racemic mixtures, and the hydrolysis was followed in each case almost to completion. As others have noted (7-9), both optical forms of the glycyll peptides were hydrolyzed. With extracts of liver and hepatoma, the rates of hydrolysis of the two antipodes of glycyll-DL-alanine and glycyll-DL-phenylalanine were so different that it could be assumed that the more susceptible isomer was

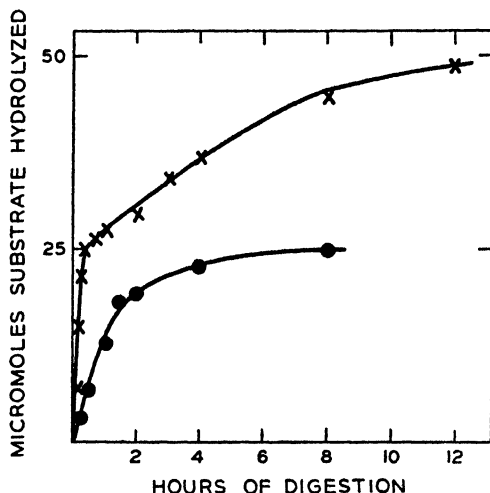


FIG. 1. Hydrolysis curves of 0.05 M glycyll-DL-alanine (X) and chloroacetyl-DL-alanine (●) with rat liver extracts containing 0.4 and 1.1 mg. of N per cc., respectively. The actual rate value for hydrolysis of the more susceptible isomer of glycyll-DL-alanine (Table I) was calculated from a curve obtained with a more dilute extract.

completely hydrolyzed before there was appreciable hydrolysis of the less susceptible isomer (Fig. 1).⁴ In such cases, it was necessary to employ higher extract concentrations to follow the hydrolysis of the less susceptible than for the more susceptible isomer. With kidney extracts, the rate of hydrolysis of the more susceptible isomer of glycyll-DL-phenylalanine is also considerably greater than that of the less susceptible isomer. How-

³ Under our conditions, the ninhydrin procedure results in a negligible hydrolysis of all the peptides studied except glycyllglycine, which is split to a few per cent (*cf.* (5)).

⁴ By the "more susceptible isomer" we refer to that member of the pair of antipodal peptides which is more rapidly hydrolyzed, without commitment as to whether the two isomers are hydrolyzed by the same or different enzymes.

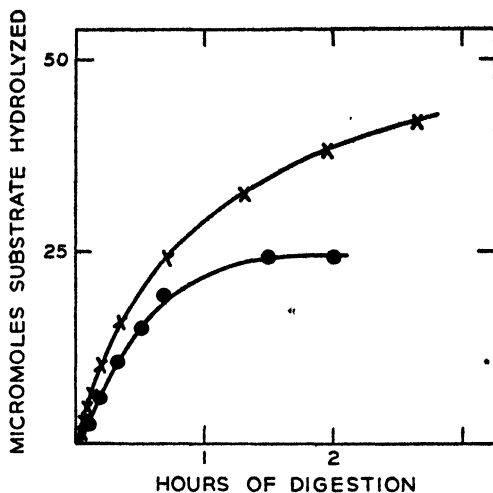


FIG. 2. Hydrolysis curves of 0.05 M glycyl-DL-alanine (X) and chloroacetyl-DL-alanine (●) with rat kidney extracts containing 0.08 and 0.6 mg. of N per cc., respectively.

TABLE I

Rates of Hydrolysis of Saturated and Unsaturated Peptides with Rat Tissue Extracts

Substrate	Micromoles $\times 10$ substrate hydrolyzed per hr. per mg. N in extract of		
	Kidney*	Liver	Hepatoma†
Glycyldehydroalanine.....	1,620	60	380
Glycyl-DL-alanine (one optical form) . . .	6,000	2000	1200
" (2nd " ").. . .	1,020	76	23
Glycyldehydrophenylalanine	520	12	0
Glycyl-DL-phenylalanine (one optical form)	10,300	3200	2120‡
" (2nd " ")	360	20	11
Chloroacetyldehydroalanine	100	28	0
Chloroacetyl-DL-alanine (one optical form)	600§	132	75

* Glycylglycine is hydrolyzed with a rate value of about 400; chloroacetylglycine is not hydrolyzed.

† Primary tumors induced in Osborne-Mendel rats by feeding *p*-dimethylaminoazobenzene; donated by Dr. J. White. Necrotic areas removed.

‡ The corresponding value when 0.025 M glycyl-L-phenylalanine was used was 2300.

§ Acetyl-DL-alanine (only one optical form) is hydrolyzed at a rate value of 310, compared with that of 18 for acetyldehydroalanine. Chloroacetyl-DL-phenylalanine (only one optical form) is hydrolyzed at a rate value of 1200, compared with that of 0 for chloroacetyldehydrophenylalanine.

ever, in the case of glycyl-DL-alanine, the hydrolysis of both optical isomers more nearly approaches that of the other, and the rate of hydrolysis of each isomer can only be roughly approximated (Fig. 2). Only one optical form of chloroacetyl-DL-alanine (Figs. 1 and 2), of acetyl-DL-alanine, and of chloroacetyl-DL-phenylalanine was hydrolyzed.

The initial reaction rates for the hydrolysis of the peptides were approximated from the slope of the respective curves (micromoles of substrate hydrolyzed plotted against time); the data are given in Table I.

In the absence of definitive information as to which optical isomer of the racemic peptides was being hydrolyzed, no designation can be made at this time, but presumably the susceptible isomer of acetyl-DL-alanine, chloroacetyl-DL-alanine, and chloroacetyl-DL-phenylalanine and the more susceptible isomer of the glycyl peptides is the L, or "natural" form. The nearly equal rates of hydrolysis of glycyl-L-phenylalanine and of the more susceptible isomer of glycyl-DL-phenylalanine support this possibility (Table I). The rates noted for the saturated peptides may be minimum for the most part, (a) since they were obtained on compounds hydrolyzing in the presence of their optical enantiomorphs, which might exert an inhibitory influence, and (b) since no possible activator was added (8, 10). Nevertheless, the rates of hydrolysis of the more susceptible forms of the saturated peptides are in every case considerably greater than those of the analogous dehydropeptides (Table I).

DISCUSSION

Bergmann and Schleich (1) showed that purified preparations of dipeptidase, aminopeptidase, and carboxypeptidase, which were highly active toward saturated peptides, had no effect upon unsaturated peptides. The converse has not yet been shown to be true, for no attempt has been made to prepare purified dehydropeptidases free of activity toward the saturated peptides. Nevertheless, some further evidence that the peptidases and dehydropeptidases are distinct enzyme systems may be derived from a comparative study of liver and hepatoma. The malignant transformation of a tissue may serve in effect, in the case of a number of closely related enzymes, as a fractionation procedure. Thus, in the consideration of the possible multiple nature of dehydropeptidase I (4), considerable weight was laid on the fact that, whereas the hydrolysis rate of glycyldehydroalanine and alanyldehydroalanine increased very considerably when liver was transformed into a hepatoma, the hydrolysis rate of glycyldehydrophenylalanine and N-methylglycyldehydroalanine decreased. Assuming that the cancerous transformation of a tissue does not result in qualitative changes in the enzymes concerned, the enrichment in activity toward glycyldehydroalanine and the concomitant decrease in activity toward glycyldehydrophenylalanine (4) (Table I) strongly suggest that the hydrolysis of these substrates is catalyzed by different enzymes. The fact that the malig-

nant transformation of liver is accompanied by a decrease in the rate whereby both optical forms of glycyl-DL-alanine are hydrolyzed (Table I) also suggests that glycyldehydroalanine and glycyl-DL-alanine are hydrolyzed by different enzyme systems. It is of some interest to note that, per mg. of tissue N, the hydrolysis rate of those saturated peptides studied, namely glycyl-DL-alanine, glycyl-DL-phenylalanine, and chloroacetyl-DL-alanine, decreases when liver becomes neoplastic (Table I), whereas it has been reported that the rate of hydrolysis of the more susceptible isomer of DL-leucylglycine and of DL-alanylglycine increases (11, 12).⁵ This difference suggests that alanylglycine and glycylalanine may be attacked by different peptidases, a possibility which had been advanced on other grounds for the corresponding D peptides by Maschmann (8). In any event, the use of carefully defined cancer tissue in comparative studies with the normal tissue of origin is sometimes capable of contributing to the solution of certain biochemical problems of fundamental interest (*cf.* (13)).

Still another suggestion that the peptidases and dehydropeptidases may be distinct systems arises from the fact that, whereas the dehydropeptides of alanine are hydrolyzed in tissue extracts much faster than the dehydropeptides of phenylalanine, the reverse holds true for the more susceptible (but not for the less susceptible) antipodes of the corresponding saturated peptides (4) (Table I).

The hydrolysis rates of the susceptible optical isomer of acetyl-DL-alanine, chloroacetyl-DL-alanine, and chloroacetyl-DL-phenylalanine, and of the more susceptible isomer of glycyl-DL-alanine and glycyl-DL-phenylalanine, are all greater than those of the corresponding analogous dehydropeptides in the same tissue extract (Table I). Little can be said in this connection of the less susceptible antipodes of the saturated peptides. Those of acetyl-DL-alanine, chloroacetyl-DL-alanine, and chloroacetyl-DL-phenylalanine are not hydrolyzed at all, while those of glycyl-DL-alanine and glycyl-DL-phenylalanine in kidney and liver extracts are apparently hydrolyzed at rates approximating those of the corresponding dehydropeptides (Table I). Further investigations will lay emphasis on the use of optically pure L and D peptides, which will also permit the use of more accurate kinetic treatment.

The only saturated peptide studied which was not hydrolyzed by aqueous extracts of kidney was chloroacetylglycine (Table I). This is in contrast to the ease of hydrolysis of the susceptible isomer of chloroacetyl-DL-alanine. Glycylglycine was hydrolyzed at a rate approximating that of glycylamino-

⁵ We have also found that, per mg. of tissue N, the more susceptible isomer of DL-alanylglycine is hydrolyzed more rapidly in hepatoma than in normal liver extracts. Under experimental conditions identical with those used for glycylalanine (Table I), the hydrolysis rate for alanylglycine in hepatoma is 3700, in liver 1630. The second optical isomer of DL-alanylglycine does not appear to be hydrolyzed under these conditions, either in liver or in hepatoma extracts.

isobutyric acid (14), which was considerably less than that of glycyl-DL-alanine. It may be suggested that the presence of a center of optical asymmetry in a favorable position within the peptide is frequently conducive to increased susceptibility to peptidase action.

SUMMARY

The rates of hydrolysis in fresh, aqueous rat tissue extracts were determined for glycyl-DL-alanine, glycyl-DL-phenylalanine, chloroacetyl-DL-alanine, acetyl-DL-alanine, and chloroacetyl-DL-phenylalanine, and the rates compared both among themselves and with those of the corresponding analogous dehydropeptides. Both optical isomers of the glycyl peptides of alanine and phenylalanine were hydrolyzed, one at a faster rate than the other, but only one isomer of the chloroacetyl and acetyl peptides was hydrolyzed. Chloroacetyl-glycine was not hydrolyzed, and glycyl-glycine was hydrolyzed in kidney at a rate approximately one-fifteenth that of the more susceptible isomer of glycyl-DL-alanine.

The more susceptible isomers of the racemic saturated peptides were hydrolyzed in extracts of kidney, liver, and hepatoma at considerably faster rates than those of the corresponding dehydropeptides. The neoplastic transformation of liver results in a decrease in the hydrolysis rate of glycyl-alanine, which is in contrast with the increase in hydrolysis rate of glycyl-dehydroalanine. The more susceptible antipodes of the saturated peptides of phenylalanine were hydrolyzed in kidney, liver, and hepatoma at a faster rate than those of alanine, whereas the converse held true of the corresponding dehydropeptides. The question of the separate identity of the peptidases and dehydropeptidases was discussed.

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THE REVERSIBLE INHIBITION OF ACETYLESTERASE BY DIISOPROPYL FLUOROPHOSPHATE AND TETRAETHYL PYROPHOSPHATE*

BY EUGENE F. JANSEN, M.-D. FELLOWS NUTTING, AND A. K. BALLS

(From the Enzyme Research Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture, Albany, California)

(Received for publication, June 2, 1948)

Diisopropyl fluorophosphate (DFP) has been shown to be a remarkably potent inhibitor of the enzymatic hydrolysis of acetylcholine (1). As a result of a wide survey (2), inhibition by dialkyl fluorophosphates appears to be specific for certain esterases and lipases (kidney acid phosphatase was found to be inhibited by relatively high concentrations). Since the cholinesterase of mammalian brain and nerve tissue is narrowly specific for acetylcholine and acetyl- β -methylcholine (3), it is often called "true" cholinesterase. Other esterases of wider specificity (those hydrolyzing tributyrin, for example) also hydrolyze acetylcholine, but are frequently referred to as "pseudo" cholinesterases.

While studying the inhibition of cholinesterase by DFP *in vitro* and *in vivo*, Mazur and Bodansky (4) found that different tissue cholinesterases show differing degrees of sensitivity to DFP. These results have been interpreted by Hawkins and Mendel (5) and by us (6) as indicating that pseudocholinesterase is about 100 times as sensitive to the inhibitory action of DFP as is cholinesterase. From a correlation of toxicity with inhibition of brain cholinesterase, Nachmansohn and Feld (7) have concluded that the toxicity of DFP is very probably due to its action on "true" cholinesterase. Since dialysis or dilution of cholinesterase-DFP mixtures or of tissue cholinesterase from animals poisoned with DFP did not result in any increase in cholinesterase, the DFP inhibition of brain cholinesterase is not to be considered readily reversible (4). However, Nachmansohn *et al.* (8) have found that over a relatively short period of time the inhibition by DFP can be reversed by dilution, the extent of the reversal being dependent on temperature, time, and DFP concentration. Eserine, which inhibits cholinesterase reversibly, was found to protect the enzyme against irreversible DFP inhibition (9). Mazur (10) also demonstrated an enzyme in animal tissues capable of hydrolyzing the phosphorus-fluorine bond of alkyl fluorophosphates, so that there exists *in vivo* concurrent inhibition of cholinesterase and detoxification. Hexaethyl tetraphosphate (HETP) exerts a

* Enzyme Research Laboratory Contribution No. 113.

strong inhibitory effect similar to that of DFP on mammalian and insect cholinesterase *in vitro* and *in vivo* (11). Even smaller concentrations of HETP than of DFP were needed to cause the same inhibition.

The effect of DFP and HETP on enzymes of plant origin was studied to determine whether any of the plant enzymes were inhibited, particularly since HETP and like substances are beginning to find use as insecticides. A preliminary report of the results has been published (6). Of the enzymes studied only acetylcholinesterase (12) was found to be inhibited by DFP, HETP, or tetraethyl pyrophosphate (TEP) (the chief active constituent of HETP).

Acetylcholinesterase occurs in citrus fruit and many other higher plants and fungi.¹ It hydrolyzes best the esters of acetic acid, including both triacetin and acetylcholine. However, very high concentrations of acetylcholine were needed to realize maximum activity, and eserine was without effect on this enzyme.

A study has been made of the conditions necessary for the inhibition of (plant) acetylcholinesterase, and of the kinetics of that inhibition. It was further observed that, when acetylcholinesterase preparations were inhibited by TEP or HETP, they spontaneously though slowly regenerated *in vitro*. Purified preparations of another enzyme from orange flavedo (pectinesterase) frequently carried some material which accelerated this regeneration. On the other hand, enzyme inhibited by DFP has not been observed to regenerate *in vitro*, though evidence was obtained that, when a whole orange was gassed with DFP, marked inhibition of acetylcholinesterase and also some subsequent regeneration thereof occurred in the intact fruit.

EXPERIMENTAL

Methods and Materials

Enzymes and Assay Methods—The following enzymes and assay methods were employed: Arlington jack bean urease was assayed according to the aeration-titration method of Van Slyke and Archibald (13), papain salt paste (14) according to the milk-clotting method of Balls and Hoover (15), crystalline β -amylase (16) according to the Schwimmer modification (17) of the Kneen and Sandstedt method (18), crude pectinesterase concentrate according to the method of continuous titration at constant pH (19), and purified acetylcholinesterase according to a similar continuous titration method previously reported with diacetin or triacetin as a substrate (12), or by a colorimetric method recently developed with *o*-nitrophenyl acetate as substrate.

The colorimetric method for acetylcholinesterase permitted the use of a tenth as much enzyme as was needed for the titrimetric method. It consisted

¹ MacDonnell, L. R., Jang, R., Jansen, E. F., and Lineweaver, H., in preparation.

in measuring the rate of formation of *o*-nitrophenol at pH 6.5 and 25°, with a No. 42 filter in a Klett-Summerson colorimeter with the test-tube adapter in place. The substrate was dissolved in alcohol and added to the enzyme solution in 0.1 M phosphate buffer at pH 6.5 (below the surface) to give a final concentration of 2 per cent alcohol and 0.003 M *o*-nitrophenyl acetate. The color formed was measured as a function of time. The rate of formation under these conditions was linear and was directly proportional to the amount of enzyme. A standard curve related color to mm of *o*-nitrophenol. The activities were expressed in millimoles of *o*-nitrophenol formed per minute per aliquot of enzyme. These activities agreed with those determined with triacetin as a substrate to within 10 per cent. The small substrate concentration needed to show the full activity of the enzyme present is noteworthy (concentrations of *o*-nitrophenyl acetate greater than 0.003 M gave the same specific activities). This concentration is less than 0.01 of that necessary for full activity with the acetins as substrates (12). Hence acetylsterase has a much greater affinity for phenyl acetates than for aliphatic acetates.

Inhibitors—The HETP² was a commercial product known to be a mixture, the DFP³ was analytically pure, the TEP⁴ was 95 per cent pure, and no analytical figures were available for the ethyl metaphosphate (EMP).⁴ Stock solutions of the inhibitors were made to be 0.01 to 0.02 M in anhydrous isopropanol. These solutions kept for several months in the refrigerator with no decrease in inhibitory power. Aliquots of these solutions sufficient to give the desired concentration of inhibitor were added to an enzyme solution and incubated at room temperature (25° ± 1°) for 20 minutes (unless otherwise specified) prior to assay. It is shown later that an incubation period is necessary. As a control, pure isopropanol was added in a corresponding amount to a similar enzyme solution in order to ascertain whether any of the inhibition observed might be due to the isopropanol. In no case did the amount of isopropanol used produce any inhibition over the time of the experiment. The term pK is used to denote the negative logarithm of the concentration of inhibitor required to produce 50 per cent inhibition under the described conditions.

Results

Effect of DFP on Several Plant Enzymes—The effect of DFP on papain, pectinesterase, urease, crystalline β -amylase, and citrus acetylsterase is

² Obtained through the courtesy of the Monsanto Chemical Company, St. Louis, Missouri.

³ Obtained from the Medical Division, Army Chemical Center at Edgewood, Maryland, through the courtesy of Captain James A. Campbell.

⁴ Kindly supplied by Dr. Howard Adler of the Victor Chemical Works, Chicago, Illinois.

given in Table I. It can be seen that the arbitrary concentration of DFP employed (0.001 M) had little or no effect on any of the enzymes tried except acetylerase and possibly papain. (The pH had no effect on DFP inhibition, as will be seen below; therefore, it was not possible that the failure of DFP to inhibit was due to pH differences.) Increasing the concentration of DFP in papain solution 3-fold caused an inhibition of 25 per cent. This inhibition was not dependent upon time, since 30 and 90 minute incubation periods caused the same inhibition, contrary to the effect observed in the inhibition of acetylerase (see below). Since the concentrations of DFP necessary for papain inhibition were so large, the effect was not pursued any further. Urease and papain depend on —SH groups for activity. Since DFP failed to cause a marked inhibition of these enzymes,

TABLE I
Effect of 0.001 M DFP on Several Plant Enzymes

Enzyme	pH	Incubation time*	Per cent inhibition
		min.	
Papain†	4.7	30	9
"	4.7	180	9
Pectinesterase . .	5.6	30	0
Urease	7.0	90	0
β-Amylase	5.9	60	2
Acetylerase (citrus) . . .	6.7	7	84
" "	6.7	43	96

* The time between the addition of the DFP to the enzyme and its assay.

† No cyanide activation was used on the papain.

it is apparent that the mode of inhibition by DFP is not through reaction with —SH groups. It is of interest that pectinesterase is an esterase unaffected by DFP.

Effect of Concentration of DFP on Acetylerase—In order to determine the concentration of DFP necessary to cause 50 per cent inhibition of acetylerase, it was necessary to select an arbitrary time of reaction between the enzyme and the inhibitor. For this purpose a 20 minute incubation period was chosen. The suggestion had been made previously (12) that wheat "lipase" (20) is in reality acetylerase. Since lipase (pancreatic) was not affected by DFP,⁵ the inhibition of the wheat enzyme would offer additional evidence of the identity of this enzyme with acetylerase. A concentrate of the wheat enzyme was made by extraction of 100 gm. of wheat germ overnight with 1 liter of 2.5 N sodium chloride at pH 7.0. The filtered extract was made 0.7 saturated with ammonium sulfate and the

⁵ Unpublished results.

precipitate was filtered off and taken up in 75 ml. of water and dialyzed in the cold. The resulting solution had an activity of [AE u.] ml. = 0.002. Aliquots of this solution as well as those of citrus acetylcholinesterase were subjected to DFP. From Table II it is apparent that the concentration of DFP necessary for 50 per cent inhibition under the conditions of the experiment was approximately 5×10^{-5} M ($pK = 4.3$) for both preparations. This concentration is of the same order as that necessary for 50 per cent inhibition of true cholinesterase (4, 21). The fact that the same concentration of DFP caused a similar inhibition of the enzymes from both sources is additional evidence that the wheat enzyme is in fact acetylcholinesterase. That the inhibition by DFP was not due to its hydrolysis to fluoride ion was shown by the failure to obtain inhibition of acetylcholinesterase by incubation with 0.001 M NaF.

Citrus flavado press-juice contains considerable acetylcholinesterase (12). The concentration of DFP necessary to cause 50 per cent inhibition in a similar

TABLE II
Effect of DFP Concentration on Acetylcholinesterase

DFP concentration $\times 10^4$	Per cent inhibition*	
	Citrus acetylcholinesterase	Wheat acetylcholinesterase
5	86	91
1	72	75
0.5	56	65

* After 20 minutes prior exposure of the enzyme to DFP.

incubation period was found to be approximately 10 times as much as was needed for the partially purified acetylcholinesterase used above. On purification of the acetylcholinesterase (12) this difference became negligible. The explanation of these observations is still obscure, but obviously may be connected with the observations on regeneration of the inhibited enzyme.

Kinetics of DFP Inhibition—A study was made of the rate of inhibition of citrus acetylcholinesterase by DFP at 25°. The reaction was found to be bimolecular over the concentration range studied, since the product of the time necessary to cause 50 per cent inhibition multiplied by the inhibitor concentration gave a constant (Table III), except for the lowest concentration studied. Furthermore, a plot of the reciprocal of residual activity against time gave a linear function for more than half of the reaction. This may be considered as additional evidence that the inhibiting reaction is bimolecular. The DFP concentrations used were all above those with which Nachmansohn *et al.* (8) were able to obtain reversible inhibition, so that it seems unlikely that the change from reversible to irreversible inhibition

influenced these results. Hence in order to define DFP inhibition with acetylerase the time as well as the concentration must be considered. The inhibition of cholinesterase has likewise been shown to be a reaction dependent upon time (8, 22).

Non-Dependence of DFP Inhibition on pH—Aliquots of wheat acetylerase concentrate were adjusted to pH 4.9, 6.2, and 7.4, and incubated with 5×10^{-5} M DFP. The amount of inhibition observed was 50, 56, and 53 per cent, respectively. The small differences are not considered to be significant. Similarly, acetylerase in citrus press-juice at pH 5.4 and 6.1 was inhibited under like conditions to the extent of 17 and 20 per cent, respectively. Hence over the range of pH studied the reaction of DFP with acetylerase was not dependent upon pH.

Effect of Eserine and Substrate on DFP Inhibition—The fact that eserine did not inhibit the hydrolysis of acetylcholine bromide by citrus acetyl-

TABLE III
DFP Inhibition, a Bimolecular Reaction

DFP concentration $\times 10^4$	Time of 50 per cent inhibition ($t_{\frac{1}{2}}$)	$t_{\frac{1}{2}} \times$ concentration $\times 10^4$
	<i>min.</i>	
1	83	8.3
5	24	10
10	12	12
100	1	10

esterase has previously been demonstrated (12). Wheat acetylerase was likewise found to hydrolyze acetylcholine bromide; however, at a concentration of 0.9 M the relative rate of hydrolysis (on the basis of triacetin activity as unity) was only 60 per cent of that of citrus acetylerase. Incubation of wheat acetylerase at 25° for 90 minutes in 1×10^{-4} M eserine failed to show any inhibition of acetylcholine hydrolysis on subsequent assay. Moreover, the hydrolysis of acetylcholine by the wheat enzyme in the presence of 1×10^{-3} M eserine proceeded at the same rate as in the absence of eserine. Treatment with 5×10^{-5} M DFP of wheat acetylerase, which had already been incubated with 1×10^{-4} M eserine for 90 minutes, caused the same inhibition as was observed in the absence of eserine. Therefore, unlike cholinesterase, acetylerase was not inhibited by eserine and, probably for the same reason, the eserine did not protect acetylerase from DFP inhibition. Conversely, Webb (2) found that the enzymes other than cholinesterase which were inhibited by DFP were sensitive to eserine, although there was no direct correlation between the sensitivity to DFP and eserine.

In the presence of substrate the inhibition of acetylcholinesterase by DFP is greatly retarded; *i.e.*, in the presence of 5 per cent triacetin, 40 minutes were needed to reduce the activity of the wheat enzyme to one-half by the action of 1×10^{-3} M DFP, whereas in the absence of the triacetin this degree of inhibition would have occurred in 1 minute. The time needed to reduce the activity to one-half in the presence of substrate was approximately the same at lower concentrations of DFP (1 and 5×10^{-4} M). It is probable, therefore, that the point of attack of the DFP involves the point of attachment of the enzyme to the substrate.

Effect of HETP, TEP, and EMP on Acetylcholinesterase—HETP, a mixture of ethyl phosphates, was found to inhibit acetylcholinesterase (Table IV) in even smaller concentrations than were needed with DFP (Table II); approximately one-fiftieth to one-twenty-fifth as much caused 50 per cent inhi-

TABLE IV
Effect of HETP, TEP, and EMP on Acetylcholinesterase

Enzyme source	Inhibitor	Inhibitor concentration	Per cent inhibition*
		M	
Wheat	HETP	1×10^{-6}	57
"	"	1×10^{-6}	43
Citrus, purified	"	2×10^{-6}	50
"	TEP	1×10^{-6}	46
"	EMP	1×10^{-5}	50

* After 30 minutes prior exposure of the enzyme to the inhibitor.

bition in a like time. This difference is considerably greater than that observed for cholinesterase (11). The active insecticidal constituent of HETP,⁶ TEP, was just as effective on a molar basis. However, EMP, the other major constituent of HETP, was one-tenth as active as TEP. Since the EMP was impure and probably still contained residual TEP, the inhibition was probably due to the latter. Here again the reaction between HETP and acetylcholinesterase was found to be bimolecular. Acetylcholinesterase in citrus press-juice required 10 times as much TEP to give 50 per cent inhibition as did the partially purified acetylcholinesterase.

Regeneration of Acetylcholinesterase—As cited earlier, the conclusion that cholinesterase is irreversibly inhibited by DFP is based largely on the behavior of the inhibited enzyme upon dialysis. Similar experiments with inhibited acetylcholinesterase showed that dialysis, even after 11 days, produced no restoration of activity when the enzyme (from either wheat germ or orange

⁶ Private communication from Dr. H. L. Haller, Bureau of Entomology and Plant Quarantine, United States Department of Agriculture.

flavado) was inhibited with DFP.⁷ When it was inhibited by TEP or HETP, however, a small but definite increase was observed. (For example, a wheat germ preparation inhibited to 14 per cent of its original activity by 1×10^{-5} M HETP rose to 17 per cent in 3 days; a citrus preparation, inhibited to 16 per cent of its original activity by 1×10^{-4} M TEP, rose to 30 per cent in eleven days.)

This regeneration of activity, however, was found not to depend upon dialysis, but to occur simply on standing. As shown in Table V, the reappearance of activity was more rapid at room temperature than at 5°. However, in the long run, greater regeneration occurred at the lower tem-

TABLE V
Regeneration of TEP-Inhibited Acetylerase

Inhibitor*	Added pectinesterase preparation†	Temperature‡	Per cent of original activity after				
			4 hrs.	1 day	5 days	14 days	20 days
		°C.					
DFP	—	5		21	15	5	3
TEP	—	5	1		5	34	45
"	+	5	3	7	27	54	
"	+	25	3	23	29	29	
"	+ (heated)§	5	2	2		32	34

* The inhibitor was added to the acetylerase to give a concentration of 1×10^{-4} M.

† A purified, lyophilized preparation of citrus pectinesterase¹ was added to the acetylerase to give a concentration of 0.7 pectinesterase unit per ml.

‡ After an incubation period of 4 hours at 25°, the reaction mixtures were stored at the respective temperatures.

§ A solution of the pectinesterase preparation was heated to 60° for 5 minutes and cooled prior to its addition to the acetylerase.

perature. The results were by no means regular and indicated to us the likelihood that some unidentified factor in the preparations was participating in the observed return of activity. This supposition was strengthened by the observation that additions of purified citrus pectinesterase frequently (but not always) increased the rate of regeneration. A preparation of tomato pectinesterase, however, was without effect. The pectinesterase content of the preparations used did not appear to be correlated with their regenerative effect on inhibited (citrus) acetylerase. Heated pectinesterase preparations were entirely inactive in this respect.

While preparations of citrus acetylerase in aqueous solution were never

⁷ The dialyses were carried out in the cold with conditions under which acetylerase was stable; *i.e.*, dialysis against 0.1 M sodium oxalate (12).

observed to recover from inhibition by DFP, the enzyme was found to be regenerated *in situ* after oranges were gassed with DFP vapor. It has previously been shown that the major portion of acetylcholinesterase occurs in the flavedo of orange rind (12). When oranges are evacuated in a desiccator containing a small amount of DFP, some of the chemical is volatilized and forced into the fruit when atmospheric pressure is restored. By repeating the evacuation several times, it was found that over 95 per cent of the acetylcholinesterase in the outer layers of the rind could be inhibited.

TABLE VI
*Regeneration of DFP-Inhibited Citrus Acetylcholinesterase, in Situ**

Rind part†	Per cent of untreated activity			
	After treatment	After oranges stood at 25°		
		1 day	3 days	4 days
Whole flavedo	4	13		
“ “	5			40
Outer “†	5		15	
Inner “§	5		26	
Albedo	50		28	

* The acetylcholinesterase was inhibited by evacuating the oranges in a desiccator containing 1 ml. of DFP for 1 minute, then allowing the oranges to remain at atmospheric pressure for 1 minute and repeating this cycle ten times. After the last evacuation the oranges were allowed to remain in the desiccator at atmospheric pressure for at least 0.5 hour. Washing the oranges after treatment had no effect on the inhibition.

† The rind parts were allowed to remain on the orange for the time indicated in the last column, after which they were removed and extracted as previously described (12).

‡ The outer flavedo was that part of the flavedo which contained most of the oil, and was removed by grating.

§ The inner flavedo was that part of the flavedo which contained little oil but still relatively large amounts of pigment.

However, if such fruit were allowed to stand 3 to 4 days at 25°, the acetylcholinesterase in the flavedo was found to have regenerated to the extent of 15 to 40 per cent of its original (uninhibited) value (Table VI). In the meantime, the rind apparently suffered some autolytic changes, but the replacement of the esterase activity can hardly be attributed to diffusion from elsewhere in the fruit, for in the layers of tissue beneath the outer flavedo (as seen in Table VI) about the same degree of regeneration was observed. Consequently there exist some factors in citrus fruit capable of forming active acetylcholinesterase after that enzyme has been inhibited by either DFP or TEP. Attempts to concentrate or identify such factors have so far met

with no success, but they will be continued for the sake of their importance, not only to certain problems of citrus metabolism but also to that of recovery from DFP poisoning.

DISCUSSION

Certain properties of plant acetylerase as it occurs in fairly crude preparations from wheat germ and orange flavedo may now be compared with those of the cholinesterases of animal tissue. The comparisons are summarized in Table VII. The general similarity between the plant and animal enzymes is striking. In both cases, the action of the phosphate inhibitors appears, at the present writing, to be a specific one. While this similarity is backed by a great amount of experimental work, it is nevertheless based on ignorance, for the possibilities are not nearly exhausted. At present the known differences are more profitably discussed.

TABLE VII
Comparison of Acetylerase with Cholinesterase

Property	Cholinesterase		Acetylerase
	True	Pseudo	
Affinity for acetylcholine	Great	Less	Least ($K_m = 1.6 \text{ M}$)
Inhibition by eserine	+	+	0
Specificity	Narrow	Wide	Wide
pK of DFP inhibition	3.8-5 (20)	6.5-7 (20)	4.3
" " HETP "	7-7.5 (10)	7 (10)	6

The figures in parentheses are bibliographic references

The important rôle of acetylerase in plant metabolism can hardly be the hydrolysis of acetylcholine, and the observed similarities between this enzyme and the cholinesterases are more probably dependent upon the ester-hydrolyzing mechanism of all of them than upon any factor specific for choline. This is supported by the wider specificity of the plant enzyme. It should be noted also that not aliphatic, but aromatic, acetates are its preferred substrates. Thus the affinities (K_m values) of acetylerase for acetylcholine, triacetin, and *o*-nitrophenyl acetate were 1.6 M, 0.03 M (12), and 0.001 M, respectively.

The inhibition constants (pK) for HETP are practically the same, but those for DFP differ widely. This suggests that the mode of action of HETP inhibition may be quite different from that of DFP inhibition.*

* The behavior of plant acetylerase undoubtedly resembles that of animal pseudocholinesterase.

There is a marked difference in the concentration of DFP required for the inhibition of the three enzymes shown in Table VII, but no difference in the concentration required of HETP. This again suggests that the mode of the two inhibitions may be different, a supposition that is borne out by the observation that the plant enzyme, while it recovers from both inhibitions, does so under totally different circumstances. The reappearance of the HETP-inhibited enzyme depends upon an unknown factor in the water-soluble preparations. The factor is evidently heat-labile and operates faster but not more extensively at higher temperatures. These properties are characteristics of an enzyme. After being inhibited by DFP, acetylsterase reappears only when in contact with the original tissue, and during a process outwardly resembling autolysis of that tissue. It has not been technically possible to inhibit citrus acetylsterase by HETP *in situ* apparently because of the difficulty of volatilizing enough of the chemical. Application of a solution to the surface carries with it the possibility of adsorption, and unsatisfactory penetration to the interior. In such experiments, however, little inhibition of the flavedo enzyme was obtained. Perhaps the solid tissues are so much richer in the regenerating factor than are extracts thereof that the HETP-inhibited enzyme is almost immediately regenerated, and even the DFP-inhibited enzyme recovers slowly. Otherwise the two kinds of recovery must differ fundamentally.

SUMMARY

1. A colorimetric method for the assay of acetylsterase based on the rate of liberation of *o*-nitrophenol from *o*-nitrophenyl acetate has been developed. The affinity of acetylsterase for this substrate was found to be much greater than that for acetic esters of aliphatic alcohols.

2. Of the enzymes of plant origin, urease, papain, crystalline β -amylase, pectinesterase, and citrus acetylsterase, only the acetylsterase was found to be appreciably inhibited by DFP. The pK value for a partially purified citrus acetylsterase was found to be 4.3. For a wheat germ concentrate, the pK value was the same, thus giving additional evidence that the wheat enzyme is acetylsterase and not lipase. Fluoride ion was without effect on acetylsterase; hence the effect of DFP was not due to its hydrolysis to fluoride ion.

The acetylsterase in citrus flavedo press-juice required approximately 10 times as much DFP to cause 50 per cent inhibition as did the enzyme after partial purification from this source.

3. A study of the kinetics of inhibition by DFP showed the reaction to be bimolecular. Hence, to define DFP inhibition with acetylsterase, the time as well as the concentration must be considered.

4. The inhibition of acetylcholinesterase by DFP was found to be independent of the pH of the reaction mixture over the range of stability of the enzyme (pH 4.9 to 7.4).

5. Eserine was without effect on the hydrolysis of acetylcholine by acetylcholinesterase, and likewise did not protect the enzyme from DFP inhibition.

In the presence of substrate (5 per cent triacetin) 1×10^{-3} M DFP required 40 minutes to reduce the activity to one-half, whereas in the absence of substrate this amount of inhibition occurred in 1 minute. In the presence of substrate at lower DFP concentrations, this same decrease occurred in approximately the same time. Hence the point of attack of the DFP must involve the site of attachment of the enzyme to the substrate.

6. The pK value for inhibition of acetylcholinesterase by HETP (a mixture) was found to be one-fiftieth to one-twenty-fifth of the value for inhibition by DFP. TEP, the active insecticidal constituent of the mixture, was just as effective on a molar basis. EMP, the other major constituent of HETP, was considerably less effective. The reaction of these inhibitors with acetylcholinesterase was likewise bimolecular.

7. Dialysis of acetylcholinesterase inhibited with DFP failed to cause any regeneration of activity. TEP- or HETP-inhibited acetylcholinesterase slowly regenerated on storage at 5°. Sometimes more than 50 per cent of the original activity returned in 30 days. A preparation of citrus pectinesterase accelerated this regeneration.

8. Citrus acetylcholinesterase inhibited *in situ* by DFP was found to "regenerate" when the intact fruit was allowed to stand for 3 to 4 days. Hence factors responsible for the regeneration of DFP- and TEP-inhibited acetylcholinesterase exist in citrus fruit.

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LETTERS TO THE EDITORS

THE EFFECT OF DIETARY PROTEIN ON THE TURNOVER OF PHOSPHOLIPIDES, RIBONUCLEIC ACID, AND DESOXYRIBONUCLEIC ACID IN THE LIVER

Sirs:

When rats are placed on a diet free from protein but adequate in every other respect, protein, phospholipides, and ribonucleic acid (RNA) are lost from the liver, while the desoxyribonucleic acid (DNA) content does not alter significantly. Therefore, the DNA P values may be used as terms of reference, and the protein N, phospholipide P, and RNA P contents expressed as mg. per mg. of DNA P (see the table).¹

It appeared to be of interest to examine the turnover rates of the P-containing compounds at different stages of the protein-free regimen. For this purpose, one group of five male hooded rats 3 to 4 months old was fed on a diet containing 23 per cent casein for 7 days (Diet I), a second group of four rats on the same diet for 7 days and then on a protein-free diet for 1 day (Diet II), a third group of four rats on a protein-free diet for 4 days (Diet III), and a fourth group of five rats on a protein-free diet for 7 days (Diet IV). The average body weight at the beginning of the dietary periods was 294 gm., varying between 253 and 356 gm. The daily food intake was 5.8 gm. per 100 gm. of body weight on Diets I and II, and 4.8 gm. on Diets III and IV. P^{32} (18 to 20 microcuries) was injected subcutaneously 6 hours before the rats were killed for the analysis of liver tissue. The ratio of liver phospholipide (or RNA or DNA) $P^{32}:P^{31}$ to liver inorganic $P^{32}:P^{31}$ (= relative specific activity) was used as a measure of the turnover rate.² In order to obtain an indication of the total turnover of phospholipide (or RNA) P in the liver, the ratio of phospholipide (or RNA) $P^{32}:DNA P^{31}$ to inorganic $P^{32}:P^{31}$ (= total relative activity) was calculated. In this ratio DNA P^{31} , which remains unaffected by changes in dietary protein, replaces phospholipide P^{31} or RNA P^{31} .

During the first few days on the protein-free diet, the relative specific activities of phospholipide P and RNA P increased, while that of DNA P

¹ Kosterlitz, H. W., *Nature*, **154**, 207 (1944); *J. Physiol.*, **106**, 194 (1947). Campbell, R. M., and Kosterlitz, H. W., *J. Physiol.*, **106**, 12 P (1947).

² Hevesy, G., in Nord, F. F., and Werkman, C. H., *Advances in enzymology and related subjects*, New York, **7**, 111 (1947).

decreased. The fact that the total relative activities of phospholipide P and RNA P showed no significant change, with the exception of the 7 days value, indicates that the increased rate of renewal of these substances compensated for their loss from the livers of rats fed on a protein-free diet. It would appear, therefore, that the total turnover of the phospholipides and of RNA is determined by the metabolic needs of the liver and is, at least within the limits of these experiments, independent of how much of these substances is present in the liver. The low 7 days value and other unpublished data suggest that in prolonged protein deficiency this rule

Diet No.	DNA P*	Protein N	Lipide P	RNA P	Relative specific activity $\times 100^\dagger$			Total relative activity†	
		Mg. per mg. DNA P			Lipide P	RNA P	DNA P	Lipide P	RNA P
	<i>mg. per liver</i>								
I	2.43	131	6.24	4.94	35.8	10.4	0.78	2.23	0.51
		$\pm 2.6^\dagger$	± 0.13	± 0.10	± 1.8	± 0.2	± 0.02	± 0.09	± 0.01
II	2.37	110	5.22	4.02	41.7	12.5	0.68	2.18	0.50
		± 2.4	± 0.08	± 0.06	± 1.2	± 0.6	± 0.07	± 0.05	± 0.02
III	2.29	88	4.15	3.76	53.2	15.7	0.57	2.22	0.59
		± 2.3	± 0.05	± 0.09	± 4.2	± 0.6	± 0.05	± 0.20	± 0.03
IV	2.37	81	3.66	3.45	48.2	15.7	0.54	1.76	0.54
		± 1.7	± 0.13	± 0.11	± 1.7	± 0.6	± 0.05	± 0.09	± 0.03

* The values of DNA P have been adjusted for a body weight of 300 gm. by means of the regression equation, $Y = 0.00583X + 0.540$, in which Y stands for mg. of DNA P, and X for gm. of body weight on the last day of a diet adequate in protein. Since the standard error of the differences between the means is ± 0.103 , the differences are not significant.

† See the text.

‡ Standard error of the mean.

may no longer hold for phospholipide P, a fact which may possibly have some bearing on the development of liver injury in protein deficiency.

*Physiology Department
Marischal College
University of Aberdeen
Aberdeen, Scotland*

ROSA M. CAMPBELL³
H. W. KOSTERLITZ⁴

Received for publication, July 1, 1948

³ Carnegie Senior Scholar.

⁴ Grants provided by the Medical Research Council of Great Britain to defray part of the expenses are gratefully acknowledged. The authors wish to thank Mr. M. H. Quenouille for the statistical treatment of the DNA values. They are indebted to Miss I. Fraser for valuable technical assistance.

ON THE MODE OF ACTION OF BIOTIN*

Sirs:

In a recent communication¹ Lichstein and Umbreit have presented experiments demonstrating a function for biotin. These investigators found that incubation of freshly harvested cells of *Escherichia coli* in molar phosphate at pH 4 and 37° resulted in marked loss of their ability to produce carbon dioxide from aspartic acid. The enzymatic activity of these "in-activated" cells was restored by the addition of crystalline biotin. Their further analysis of the intermediate steps in this reaction localized the effects of biotin in the decarboxylation of oxalacetic acid. Using the same test system, these authors later showed that biotin also was involved in the deamination of L-aspartic acid as well as DL-threonine and L-serine.²

In connection with our studies on the mode of action of biotin, we attempted to duplicate these findings under identical experimental conditions.³ We were able to confirm the inactivating effect of phosphate incubation, but were entirely unsuccessful in numerous attempts to reactivate the treated cells with either biotin or a mixture of biotin with the following vitamins: riboflavin, nicotinic acid, pantothenic acid, pyridoxal, pteroyl-glutamic acid, thiamine, and *p*-aminobenzoic acid (see the table). We can offer no explanation for this difference in results.

Further experiments, summarized in the table, revealed that hot water extracts of *E. coli* and *Lactobacillus arabinosus*⁴ cells have the ability to restore the enzymatic activity of the phosphate-inactivated cells. Similar extracts prepared from *L. arabinosus* grown on a "biotin-free" medium containing aspartic acid and Tween 80 also possessed the same stimulatory activity. Since such extracts are practically devoid of biotin (as measured by *L. arabinosus* assay), it seems very unlikely that biotin plays any rôle in these enzyme systems.

In 1938, Gale⁵ had already shown that one of the deaminase systems of *E. coli* could be inactivated by incubation with water, and that the activity could be restored by hot water extracts prepared from *E. coli* cells and several other natural materials. Formate or adenosine also possessed

* Supported in part by grants from the Committee on Growth of the National Research Council.

¹ Lichstein, H. C., and Umbreit, W. W., *J. Biol. Chem.*, **170**, 329 (1947).

² Lichstein, H. C., and Umbreit, W. W., *J. Biol. Chem.*, **170**, 423 (1947).

³ We are indebted to Dr. Lichstein for the strain of *Escherichia coli* used in his experiments.

⁴ Grown on the medium of Wright and Skeggs. Wright, L. D., and Skeggs, H. R., *Proc. Soc. Exp. Biol. and Med.*, **56**, 95 (1944).

⁵ Gale, E. F., *Biochem. J.*, **32**, 1583 (1938).

similar stimulatory effects. The decreased deaminase activity of our phosphate-inactivated cells could also be restored by adenosine or a hot water extract of *E. coli* cells. However, adenosine was unable to stimulate the rate of carbon dioxide production from aspartic acid by inactivated cells. Studies on the chemical nature of the substances responsible for this latter effect are in progress.

Reactivation of Phosphate-Inactivated Cells of E. coli by Various Materials

	"Untreated" cells	Materials added to "inactivated" cells*					
		None	Biotin (500 m γ)	Biotin (500 m γ) + vitamins†	Adeno- sine (0.5 mg.)	<i>E. coli</i> extract	<i>L. arabinosus</i> extract‡
QCO ₂ (N ₂)	60	5	5	4	3	35	35
	58	0	1	1	0	48 (1.0)	32
	45	2	2	3	1	45	30 (0.012)
	45	1	0	1	2	42	28 (0.010)
	50	1	2	0	2	46	23 (0.008)
QNH ₂ ¶	28	10	8	12	28		
	45	13	9	12	33		
	50	12	10	13		27	
	56	8	6	8		26	
	56	14	15	13		28	

* The bacterial extracts were prepared by extracting the washed cells with boiling water for 10 minutes. The figures in parentheses represent the millimicrograms of biotin contained in the amount of extract added per Warburg flask.

† In the concentrations employed by Lichstein and Umbreit.¹

‡ Cells grown in the presence of biotin.

§ Cells grown in the presence of either Tween 80 or oleic acid in lieu of biotin.

|| C.mm. of carbon dioxide liberated from L-aspartic acid per mg. of dry cells per hour.

¶ C.mm. of ammonia liberated from L-aspartic acid per mg. of dry cells per hour.

Analyses of *E. coli* cells incubated for varying periods of time with molar phosphate at pH 4 have revealed no changes in the biotin content of these cells.

*Department of Chemistry, University of Pittsburgh,
and the Institute of Pathology, Western
Pennsylvania Hospital
Pittsburgh*

A. E. AXELROD
KLAUS HOFMANN
SARAH ELLEN PURVIS
MARJORIE MAYHALL

Received for publication, July 9, 1948

PROCEDURE FOR THE STUDY OF CERTAIN ENZYMES IN MINUTE AMOUNTS AND ITS APPLICATION TO THE INVESTIGATION OF CYTOSINE DEAMINASE

Sirs:

The application of filter paper chromatography¹ to the study of enzyme reactions offers interesting possibilities. Certain enzymes can be demonstrated, and in some cases assayed, by the direct mixing on the paper of

Experiment No.	Enzyme	Reaction with substrate	Time	Cytosine remaining	Uracil formed	Deamination of cytosine	
						From uracil	From NH ₃
			min.	γ	γ	per cent	per cent
1	<i>B. coli</i> , cells	On paper	30	34	9	19	
			60	31	14	28	
			120	23	22	44	
2	" " "	<i>In vitro</i>	30	24	6	17	14
			60	18	12	35	21
			120	8	23	67	56
			180	3	28	84	69
			240		32	94	76
3	" " cell-free extract	" "	30	2	32	94	
			60	0	34	101	
			120				95
4	Yeast, cell-free extract	" "	30		22	65	
			60		27	81	
5	" " "	" "	60				91
			120		33	98	
6	" " "	" "	60		45	89	
7	" " "	" " (anaerobic)	60		52	100	

exactly measured quantities (0.01 to 0.02 cc.) of enzyme and substrate, followed by incubation in a moist atmosphere, stoppage of the reaction by heating to 100°, and chromatography. Bacterial suspensions may also be studied in this manner. In other cases it is preferable to perform the enzymatic reaction *in vitro* and then to transfer portions of the mixture to paper for the identification and estimation of reaction products.

The development of microprocedures for the separation, by chromatography, and the characterization and estimation, by ultraviolet spectro-

¹ Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, **38**, 224 (1944).

photometry, of purines and pyrimidines² has made attractive the study of the enzymes active in the degradation of these compounds. Dependence upon indirect criteria of enzymatic action (liberation of NH_3 , etc.) thus is replaced by the direct estimation of both substrate and reaction product at any stage.

These studies have led to the demonstration in yeast and *B. coli* of the enzyme cytosine deaminase, catalyzing the conversion of cytosine to uracil. The accompanying table presents a few experiments which were carried out at 36° in phosphate buffer of pH 7. In Experiment 1, 0.02 cc. of an aqueous suspension containing 480 γ of bacteria (dry weight) was mixed on the paper with 50 γ of cytosine in 0.01 cc. of 0.1 M buffer. Similar proportions were employed *in vitro* in Experiment 2. Powerful cell-free extracts of the enzyme (present in the 20,000 R.P.M. supernatant) were obtained from *B. coli* and yeast, crushed in a bacterial mill (Experiments 3 to 7). The preparation tested in Experiment 5 was free of adenase and guanase.

A similar arrangement permitted the study of the conversion of adenine to hypoxanthine by *B. coli* and of guanine to xanthine by the guanase of rabbit liver. The experiments are being continued.

Department of Biochemistry
Columbia University
New York

ERWIN CHARGAFF
JACOB KREAM

Received for publication, July 14, 1948

² Vischer, E., and Chargaff, E., *J. Biol. Chem.*, **168**, 781 (1947); *Federation Proc.*, **7**, 197 (1948); *J. Biol. Chem.*, in press.

KETONE BODY FORMATION FROM TYROSINE*

Sirs:

Though tyrosine is generally considered ketogenic in animals, experimental support for this hypothesis is extremely scanty. By means of isotopic tyrosine it has now been possible to substantiate its conversion to acetoacetate by liver and to provide information concerning the mechanism of this transformation.

To 10 gm. of rat liver slices suspended in 120 ml. of phosphate-saline there were added 40 mg. of tyrosine labeled in the β -carbon¹ with C^{14} , and the mixture was incubated 2 hours in oxygen at 38°. After deproteinization with copper-lime, one-half of the medium was treated according to the Van Slyke procedure for conversion of acetoacetate to acetone and CO_2 . The acetone isolated as the Denigès complex had a high activity, whereas the CO_2 had a very low activity. Degradation of this acetone with hypiodite yielded iodoform with high activity, indicating the presence of isotope in either the α - or γ -carbon of acetoacetate, or both.

To locate the position of the isotope further, advantage was taken of our finding that acetoacetate, when treated with an excess of permanganate in the cold, is rapidly and quantitatively converted to 1 molecule each of acetic, formic, and carbonic acids. The reasonable assumption was made that these three acids are derived, respectively, from the β -, γ -, the α -, and the $COOH$ carbons. Accordingly, the second half of the medium, after addition of carrier acetoacetate, was oxidized with $KMnO_4$, the excess of $KMnO_4$ was destroyed with peroxide, and the volatile acids distilled with steam. The formic acid in the distillate was oxidized to CO_2 with mercuric sulfate and the acetate recovered by distillation and identified by its Duclaux constants.

The activities of these various degradation products (see the table below) are relative values, based on an assumed activity of 100 for the tyrosine. All measurements were made after conversion to $BaCO_3$, which was assayed as an "infinitely thick" layer under a mica window counter.²

* Financially supported by the Sun Oil Company, Philadelphia, and by a grant from the United States Public Health Service, under the National Cancer Institute Act.

¹ The DL-radiotyrosine, having an activity of 0.9 microcurie per mg., was prepared in the Radiation Laboratory of the University of California and supplied through the kindness of Dr. Melvin Calvin. For use in these experiments the sample of 2.34 mg. was diluted with 230 mg. of L-tyrosine and crystallized from water, yielding essentially pure L-tyrosine, with an activity of 678 counts per minute (after combustion and measurement as $BaCO_3$ spread in an "infinitely thick" layer over an area of 5 sq. cm.).

² Reid, A. F., in Preparation and measurement of isotopic tracers, Ann Arbor, 83 (1946).

Inasmuch as substantially all of the activity of the acetoacetate is in the α -carbon atom, we conclude that acetoacetate must arise from tyrosine by a process whereby the α - and β -carbon atoms of tyrosine respectively form the carboxyl and α -carbon atoms of acetoacetate, and two of the ring carbon

Distribution of C^{14} among Acetoacetate Carbon

Carbon of acetoacetate	Substance	Method of degradation	Specific activity
α, β, γ	Acetone	Decarboxylation	30.6
COOH	CO ₂	"	0.64
β, γ	Acetate	KMnO ₄ oxidation	1.8
α	Formate*	" "	159
	Respiratory CO ₂		1.3

* Part of the activity of this fraction came from the tyrosine itself.

atoms provide the β - and γ -carbon atoms of acetoacetate. Such a process is in complete accord with the following previously suggested sequence of steps: tyrosine to *p*-hydroxyphenylpyruvic to homogentisic to acetoacetic.³

Research Institute of Temple University
Philadelphia

SIDNEY WEINHOUSE
RUTH H. MILLINGTON

Received for publication, July 21, 1948

³ Edson, N. L., *Biochem. J.*, **29**, 2498 (1935).

OXIDATIONS BY ACETOBACTER SUBOXYDANS

Sirs:

Acetobacter suboxydans is known to bring about oxidation of polyhydroxy compounds. The action of the bacterial enzyme is limited, however, to substances with specific configurations. According to the generalization of Bertrand,¹ only those hydroxyl groups are oxidized which are situated between a primary hydroxyl group and a secondary one in a cis position. Hann, Tilden, and Hudson,² in a comparative study of D and L sugar alcohols, demonstrated that only the D form is oxidized readily. In the case of inositol isomers, even a greater stereochemical specificity is required; as was shown in a recent study by Magasanik and Chargaff,³ only hydroxyl groups in a polar position are attacked by *Acetobacter suboxydans*. We wish to report our findings on the action of *Acetobacter suboxydans* upon D-glucose dimethylacetal,⁴ which has a configuration favorable for oxidation according to the Bertrand-Hudson rule.

A comparison of the oxidation rates of various substrates reveals (see the table) that meso-inositol, D-sorbitol, calcium D-gluconate, and glycerol are oxidized rapidly, whereas D-glucose dimethylacetal consumes only an insignificant amount of oxygen. A variation of the concentration of glucose dimethylacetal between 10 and 100 micromoles per Warburg vessel did not change this result. In this connection it is interesting to note that D-mannose diethylmercaptal is also resistant to the action of *Acetobacter suboxydans*.²

Glucose dimethylacetal or glucose diethylmercaptal, when added in concentrations varying from 20 to 100 micromoles to Warburg vessels containing 20 micromoles of inositol, did not influence the rate at which inositol was oxidized. Thus, neither of these substances has an inhibitory effect on the action of the bacterial enzyme.

Attempts to adapt *Acetobacter suboxydans* to the oxidation of glucose dimethylacetal by growing the bacteria on this substrate failed. After a series of seven transfers on media containing decreasing amounts of D-sorbitol (1 to 0.05 per cent) and increasing amounts of glucose dimethylacetal (0.1 to 1 per cent), the bacteria were not able to bring about an oxidation of the latter substance.

¹ Bertrand, G., *Compt. rend. Acad.*, **126**, 762 (1898).

² Hann, R. M., Tilden, E. B., and Hudson, C. S., *J. Am. Chem. Soc.*, **60**, 1201 (1938).

³ Magasanik, B., and Chargaff, E., *J. Biol. Chem.*, **174**, 173 (1948).

⁴ Wolf from, M. L., and Waisbrot, S. W., *J. Am. Chem. Soc.*, **60**, 854 (1938).

The inability of *Acetobacter* to attack D-glucose dimethylacetal demonstrates that, besides the steric requirements expressed by the Bertrand and

Rate of Oxidation by Acetobacter suboxydans

The Warburg vessels in each experiment contained 0.5 cc. of the suspension of resting bacteria (about 10 mg. dry weight), 0.5 cc. of solution containing 20 micro-moles of substrate, and 2 cc. of 1/15 M phosphate buffer of pH 6. The experiments were carried out at 38° in the presence of air. Calculated oxygen consumption, 224 c.mm.

Time	Oxygen consumption in c.mm.				
	<i>meso</i> -Inositol	D-Sorbitol	Calcium D-gluconate	D-Glucose dimethylacetal	Glycerol
<i>min.</i>					
5	131	18	32	1	19
15	220	48	60	4	51
30	228	95	99	4	81
45	230	158	148	6	111
60	235	221	202	7	136

Hudson generalizations, additional factors are decisive for the oxidation of open chain polyhydroxy compounds.

*Banting and Best Department of Medical Research
University of Toronto
Toronto, Canada*

BEAT ISELIN¹

Received for publication, July 29, 1948

¹ The author wishes to express his gratitude to Professor H. O. L. Fischer, who made it possible for him to work on this problem.

ENZYMATIC CONDENSATION OF ACETATE TO ACETOACETATE IN LIVER EXTRACTS

Sirs:

We have observed a synthesis of acetoacetate from acetate + ATP in pigeon liver extracts. The same preparation was used as for experiments on enzymatic acetylation of sulfanilamide.¹ The synthesis of acetoacetate

TABLE I

A 1 ml. purified fraction was used per sample (corresponding to 180 mg. of dry liver powder) in a total volume of 3 ml. containing 0.01 M cysteine, 0.1 M sodium bicarbonate, and other additions as indicated; incubated for 2 hours at 38°.

	0.02 M acetate, 0.01 M ATP, Co A, 40 units per ml.	Same, but no Co A	Same, but no ATP	Same, but no acetate
Acetone,* micromoles.....	3.2, 3.3	0.1	0.2	0.3

* The trichloroacetic acid extracts were first acidified with sulfuric acid² and kept overnight at 38° for decarboxylation of acetoacetate. The fluid was then neutralized with sodium hydroxide and phosphate, and the acetone distilled by the procedure of Folin.⁴ Acetone was determined colorimetrically by the method of Behre.⁵ Analogous results were obtained with the determination procedure of Lester and Greenberg.³

TABLE II

A 1 ml. crude, aged liver extract was used per sample, containing 0.02 M sodium citrate to minimize ATP breakdown. The other components were present as indicated in Table I, except for Co A, incubated for 2 hours at 38°, and the acetone determined by the method of Behre.⁵

Co A, units per ml.....	4	8	12	16	20	30
Acetone, micromoles.....	0.8	1.3	2.2	2.7	3.1	3.6

may be observed with crude extracts² or with the fraction between 40 and 70 per cent ammonium sulfate saturation. This fraction contains the acetylating system and is rather free of interfering substances.

It appears from Table I that coenzyme A (Co A) is a component of this

¹ Lipmann, F., *J. Biol. Chem.*, **160**, 173 (1945).

² Kaplan, N. O., and Lipmann, F., *J. Biol. Chem.*, **174**, 37 (1948).

³ Greenberg, L. A., and Lester, D., *J. Biol. Chem.*, **154**, 177 (1944).

⁴ Folin, O., Laboratory manual of biological chemistry, New York and London, 5th edition, 211 (1934).

⁵ Behre, J. A., *J. Biol. Chem.*, **136**, 25 (1940).

system. The dependence of the rate of reaction on the concentration of Co A is shown in the experiment in Table II.

The close relationship of acetoacetate synthesis, *i.e.* the acetylation of acetate, and sulfanilamide acetylation is further emphasized by a strong, apparently competitive inhibition by sulfanilamide. This may indicate a common acetyl precursor.

*Biochemical Research Laboratory, Massachusetts
General Hospital, and the Department of
Biological Chemistry, Harvard
Medical School
Boston*

MORRIS SOODAK
FRITZ LIPMANN

Received for publication, July 31, 1948

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